NEUROTRANSMITTER ALTERATIONS IN HEPATIC FAILURE: INFLUENCE OF PRECURSOR DISTRIBUTION AND BLOOD-BRAIN BARRIER TRANSPORT

A Thesis submitted to

the University of Cape Town

for the Degree of

DOCTOR OF PHILOSOPHY

by

ANKE MELISA MANS

June, 1979

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FOREWORD

I should like to express my appreciation to my supervisor, Dr. Julien F. Biebuyck, for stimulating my interest in the exciting field of neurochemistry, and his continuous encouragement and advice. Without his enthusiastic involvement in all stages of the work, this study would not have been possible.

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1. Correlation of plasma and brain amino acid and putative neurotransmitter alterations during acute hepatic coma in the rat. A. M. Mans, S. J. Saunders, R. E. Kirsch and J. F. Biebuyck. J. Neurochem. 32 285-292 (1979).

2. Tryptophan transport across the blood-brain barrier during acute hepatic failure. Anke M. Mans, Julien F. Biebuyck, Stuart J. Saunders, Ralph E. Kirsch and Richard A. Hawkins. J. Neurochem. in press (1979).

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ABBREVIATIONS

The following abbreviations have been used in this thesis (scientifically accepted abbrevations (Biochem. J. [1978] 169 1-27) are not defined).

BUI	brain uptake index
cAMP	cyclic AMP
CNS	central nervous system
ECF	extracellular fluid
EEG	electroencephalogram
GABA	γ-aminobutyric acid
galN	D-galactosamine
hal	hepatic artery ligation
hep. devasc.	hepatic devascularization
5-HIAA	5-hydroxyindoleacetic acid
pcs	portacaval shunt

ABSTRACT

Hepatic encephalopathy, or the syndrome of disturbed consciousness often associated with advanced chronic or acute hepatic failure, is believed to be a biochemical disturbance of cerebral function. Since it has been shown that there is no shortage of energy intermediates in the brain, and transmission failure rather than energy failure is thought to be the primary event, investigations into the aetiology of hepatic encephalopathy have recently focussed on factors involved in neurotransmission.

This thesis describes studies of changes in some substances involved in neuroregulation in the brain, and their precursors, during chronic and acute hepatic failure in the rat. These substances can be divided into two groups: neuroregulators associated with ammonia metabolism (the amino acid neuroregulators, glutamate, glutamine, aspartate and GABA, derived from glucose), and those derived from the aromatic amino acids tryptophan, phenylalanine and tyrosine. Because of the importance of blood-brain barrier transport for the availability of the aromatic amino acids to the brain, plasma/brain amino acid relationships were also studied. The results suggested that the transport mechanism for these and the other neutral amino acids (leucine, isoleucine, valine, methionine and threonine), might be altered during hepatic failure; therefore this possibility was investigated in a separate series of experiments. The effect of continuous intravenous infusion of solutions with and without amino acids, on plasma and brain metabolites, was studied in chronic hepatic failure.

Chronic hepatic failure was studied in rats four weeks after performing an end-to-side portacaval shunt operation. These rats were pair-fed with sham-operated controls, and at the time of sampling there was no difference in body weight between the two groups. The biochemical changes observed closely resembled those seen in human chronic hepatic encephalopathy. No abnormalities in behaviour were seen during the four-week period.

Acute hepatic failure was produced in either of two ways:

(a) two-stage hepatic devascularization (portacaval shunt followed 65 hours later by hepatic artery ligation), or

(b) intravenous injection of the specific hepatotoxin galactosamine-HCl.

The surgical method (a) leads to an inactive and unresponsive condition about four hours after hepatic artery ligation and finally death after a further two to five hours. During the entire period, body temperature and blood glucose levels (which otherwise decrease markedly) were maintained within the normal ranges. The rats were sampled when unresponsive to all but severe stimuli four to seven hours after hepatic artery ligation.

Galactosamine (b) was used as an alternative in order to compare the biochemical changes in acute hepatic failure produced by two entirely different methods. The dose of galactosamine used was 2 g/kg given in two doses three to six hours apart. Thirty to 60 hours after injection the rats lapsed into a clearly distinguishable coma. Two behavioural states were defined, precoma and coma, and rats sampled from each group. The use of galactosamine had the advantage of avoiding the surgical trauma of the devascularization method. In addition, a

definite coma was produced by galactosamine-induced hepatic necrosis, which was not always evident after hepatic devascularization.

The alterations in brain and plasma metabolites during acute hepatic encephalopathy produced by the two methods were strikingly similar, confirming the assumption that these were due to the failing liver. They also closely resembled those seen in acute hepatic failure patients, suggesting that these are suitable models for the study of this condition. The metabolite changes will now be described in more detail.

Changes in brain ammonia and related metabolites were qualitatively similar during chronic and acute hepatic failure; however much larger alterations were found in the acute condition. The most marked changes were increases in brain ammonia and glutamine content. Changes in ammonia have long been implicated in the development of hepatic encephalopathy but it is now clear that it is not the primary aetiologic agent. However, because of its association with the metabolism of several important neuroactive substances, it may affect the pools of these neuroregulators and thus influence brain activity.

The increase in brain glutamine is of great interest since it may be related to various other changes which occur during hepatic failure. For example, a role for glutamine has been postulated in the mechanism of transport of the amino acids across the blood-brain barrier, which is altered during hepatic failure (see below). Glutamine is synthesized exclusively in brain glial cells which are the only structures to show morphological alterations during hepatic failure, and which surround the brain capillaries where transport of substrates into the brain occurs. In addition, glutamine may affect levels of glutamate

in the brain, as well as having some "false neurotransmitter," i.e. inhibitory action, itself. All these factors together suggest that glial metabolism may be of great importance in hepatic encephalopathy and should be further investigated, especially in view of the absence of any other information regarding localization of the events leading to disturbed brain function in this condition.

Small but significant decreases in brain glutamate or aspartate were seen in both chronic and acute hepatic failure. There is now good evidence that these substances act either as excitatory neurotransmitters or as neuromodulators, i.e. substances which alter the effectiveness of the neurotransmitters (a convenient collective term for these two groups is neuroregulators). Such a role in addition to their involvement in intermediary metabolism is made possible by functional compartmentation. There was also a possible increase in GABA during acute hepatic failure which has not been previously reported.

Thus, in general, hepatic encephalopathy was associated with decreases in the excitatory- and increases in the inhibitory neuroregulator amino acids. This imbalance between excitatory and inhibitory substances was also observed when considering the aromatic amine neuroregulators and their amino acid precursors. Other studies have shown that noradrenaline is decreased and serotonin is increased in the brain during hepatic failure. In this study large increases in the three aromatic amino acids tryptophan, tyrosine and phenylalanine, which influence synthesis of serotonin and the catecholamines, were found in the brain. Changes were more pronounced during acute hepatic failure. The increase in brain tryptophan in particular seemed to correlate well

with the degree of brain dysfunction. For example, in the galactosamine studies the increase was higher in the coma group than in the precoma group. There is much evidence that brain tryptophan content controls synthesis of serotonin, which may be involved in the regulation of states of consciousness. Thus, hydroxy-indole metabolism in the brain may be connected with the development of hepatic encephalopathy and the availability of tryptophan may play a crucial role.

Brain concentrations of the aromatic amino acids in normal rats have been shown to depend on the relative plasma concentrations of the large neutral amino acids tryptophan, tyrosine, phenylalanine, methionine, leucine, isoleucine, valine and threonine, since all these share the same transport system across the blood-brain barrier. Alterations in the plasma concentrations of these amino acids are characteristic of hepatic failure. The present study showed increased tyrosine and decreased leucine and isoleucine during chronic hepatic failure. This pattern of increased aromatic and decreased branched chain amino acids in the plasma (other workers have also found increased phenylalanine and decreased valine), has been implicated in causing increased uptake of tryptophan by the brain in chronic hepatic failure, by a reduction in competition. However in this study the total molar concentration of competitors in the plasma was in fact slightly higher after portacaval shunting. In acute hepatic failure it was even more obvious that competition effects could not account for the increase in brain tryptophan, since large increases were found in most of the plasma amino acids, including the branched chain amino acids. These findings led to the question of whether the actual transport system could be altered in hepatic failure, to account for high brain tryptophan in the

presence of increased plasma competitors. This possibility was subsequently examined as described below.

Several studies in normal rats have shown correlations between brain tryptophan content and the ratio of plasma total tryptophan to the sum of the competing amino acids. During hepatic failure, different correlations were found which emphasized the importance of non-albumin bound tryptophan, rather than total tryptophan, in affecting brain tryptophan. These results again suggested that extrapolations from normal to pathological conditions should be made with caution, especially when therapeutic measures are suggested such as the feeding of intravenous amino acids to correct plasma amino acid imbalances, with the aim of influencing brain amino acid uptake. This has been previously carried out in dogs with portacaval shunts and beneficial effects on both mental status as well as plasma amino acid patterns were obtained. These effects were attributed to the amino acid distribution in the infusion. Continuous infusion of a similar solution in the present chronic hepatic failure studies led to a normalization of both brain and plasma metabolites. However, an equal, and in the case of certain metabolites, even better response, was obtained with the control solution, containing all the ingredients but no amino acids. Such a control solution was not used in the earlier reported work on dogs. This suggested that it was the reduction of protein intake (the intravenously fed rats were not given food orally), and not the amino acid distribution in the infusion mixture, which resulted in the improvement observed. In acute hepatic failure the extremely high plasma concentrations of all amino acids measured suggested that in this condition, adding specific amino acids to the system would be of little

benefit. This became even more obvious when the alterations in tryptophan transport across the blood-brain barrier during acute hepatic failure were analyzed. Thus, in general, it appears that hepatic encephalopathy of differing aetiologies may result in widely differing plasma amino acid patterns, which should be determined in individual cases before considering infusion of amino acid solutions.

Possible alterations in the blood-brain barrier transport of tryptophan during hepatic failure were investigated using a single injection, dual isotope technique. ^{14}C-Tryptophan was injected together with a reference substance ^{3}H-water into the internal carotid artery and the rat decapitated after 15 seconds. The $^{14}C/^{3}H$ ratio was determined in samples of the cortex as well as the injected solution, and by assuming a constant value for the cerebral blood flow, the influx was calculated. With this method the extraction by brain of substances from the injectate, during a single capillary pass of the bolus through the brain, was examined. A range of tryptophan concentrations was used in order to enable calculation of the kinetic constants characterizing the transport mechanism. Influx of tryptophan was also determined in the presence of competing amino acids, by the addition of competitors to the injectate.

Tryptophan influx in rats after hepatic devascularization was found to be about twice that of normal rats at all concentrations tested. In both normal rats and those with acute hepatic failure, the transport system was found to consist of two components which were mathematically resolved. In each case one component was a low capacity saturable system which obeyed Michaelis-Menton kinetics and the other was a high capacity system which transported tryptophan in direct

proportion to concentration over the range tested. In acute hepatic failure the kinetics were altered so that the influx by both components was increased, but especially by the high capacity system. It was calculated that during hepatic failure, tryptophan entry would be higher than normal even when the high levels of competing amino acids were taken into account. The high plasma competitor concentrations during acute hepatic failure, in addition to the alterations in transport kinetics, result in a greatly reduced contribution by the saturable component to total influx, and thus markedly reduce sensitivity to the effects of further increases in competition. Tryptophan influx was found to correlate significantly with brain tryptophan content. Thus, these findings could explain the increase in brain tryptophan content in the presence of high plasma competitors during acute hepatic failure.

The alterations in transport appeared to be selective, which suggested that transport of other substrates might be normal or differently affected in hepatic failure. These studies indicate that more attention should be paid to precursor availability in the brain because of the importance of blood-brain barrier transport for several metabolic pathways. The study of possible alterations in blood-brain barrier transport may be worthwhile in other pathological conditions involving brain malfunction.

The interrelationships between the various changes in metabolites and their transport are discussed in detail, compared with observations in other metabolic comas, and a hypothesis put forward describing possible events in the development of hepatic encephalopathy,

INTRODUCTION

Hepatic encephalopathy refers to the disturbance of cerebral function often associated with advanced chronic or acute hepatic failure. The neuropsychiatric symptoms cover a wide range from a rapidly developing syndrome of delirium, convulsions and coma in fulminant hepatic necrosis, to a more gradully developing intellectual impairment leading to stupor and coma in patients with chronic liver disease.

Morphological abnormalities are usually absent in the brains of patients with fulminant hepatic encephalopathy, although cerebral oedema has been described in some cases (Ware, D'Agostino & Combes, 1971). On the other hand, encephalopathy resulting from chronic liver disease is often associated with abnormalities in protoplasmic astrocytes in the brain (Cavanagh, 1974). However, the absence of morphologic changes in acute hepatic failure and the fact that the coma in both acute and chronic conditions is potentially rapidly and fully reversible, suggest that neither clinical syndrome is based on fixed structural abnormalities of the brain. In addition, the varied manifestations of hepatic encephalopathy indicate widespread and progressive impairment of the central nervous system and do not seem to point to particular cerebral structures that may be involved. Generally therefore, hepatic encephalopathy is considered to be a metabolic, i.e. a biochemical disturbance of cerebral function. The variability of the clinical and pathological symptoms also suggests that several factors may be involved in the pathogenesis of the syndrome; and a multifactorial approach may be required in order to recognize

and allow for possible interdependence and synergistic effects of these factors.

Disturbances of consciousness may result from various alterations in the normal biochemical functioning of the central nervous system. These include modifications in the excitatory and inhibitory substances and their relationship to each other, alterations in either the production or use of the brain's energy supply, and changes in the ionic distribution and pH, i.e. in the internal surroundings of the brain. These may all eventually lead to altered neuronal membrane permeability and thus altered neuronal activity.

This thesis describes studies of some neurochemical factors that may be involved in the pathogenesis of hepatic encephalopathy, using various models of acute and chronic hepatic failure. Particular attention was paid to substances involved in brain neurotransmission, namely neurotransmitters and related metabolites, and the availability of precursors.

The finding of reduced cerebral metabolic rates of glucose and oxygen in human hepatic coma (Plum & Posner, 1972; Maiolo, Porro, Galli, Sessa & Polli, 1971) suggested that cerebral energy metabolism was impaired in these patients. However, the available studies did not clarify whether the decreased energy metabolism of the brain was a primary event or resulted from decreased neuronal activity secondary to a disturbance of function or excitability. Lowry (1975) has pointed out that normally work and fuel consumption are tightly coupled in the brain, and therefore neuronal activity must control energy expenditure and not vice versa. Several findings indicate that this is also true in states of unconsciousness. In a study of various experimental

comatose states, Derr & Zieve (1973) showed that the adenylate energy charge remained constant, and therefore concluded that the decreased oxygen consumption observed was a consequence of decreased use of ATP. It has now been established that there is no shortage of energy intermediates in experimental hepatic coma (Biebuyck, Funovics, Dedrick, Scherer & Fischer, 1974; Zieve, Nicoloff & Doizaki, 1975), and the primary event in brain malfunction during hepatic failure is thought to be failure of transmission rather than energy failure. This has also been suggested for other metabolic comas such as those due to uraemia (V. d. Noort, Eckel, Brine & Hrdlicka, 1970; Berghof, Mathäus, Otto, Ulrich, Held & Gottstein, 1970; Raskin & Fishman, 1976) and hypoglycaemia (Hinzen & Müller, 1971; Ferrendelli & Chang, 1973; Ferrendelli, 1975; Norberg, Ljunggren & Siesjö, 1975).

For this reason attention has been focused on brain neurotransmitter metabolism in hepatic encephalopathy. Altered brain concentrations of the neurotransmitters noradrenaline and serotonin (decreased and increased respectively), are characteristic findings in experimental hepatic encephalopathy (Knell, Davidson, Williams, Kantamaneni & Curzon, 1974; Dodsworth, James, Cummings & Fischer, 1974). There is also evidence of increased serotonin turnover as indicated by increased concentrations of the major metabolite 5-hydroxyindoleacetic acid in brain and cerebrospinal fluid. Noradrenaline appears to have both inhibitory and excitatory effects on various regions of the brain, but it generally produces arousal while serotonin is more consistently inhibitory (Fischer & Baldessarini, 1976).

While the biogenic amines undoubtedly play an important role in brain function, the neurons containing these substances represent only

a fraction of the total in the central nervous system (Snyder, Young, Bennet & Mulder, 1973). Therefore it is probable that the amines account for transmission at only a small proportion of central nervous synapses and that other systems are present which use different neurotransmitters. Many putative neurotransmitters have been proposed (Table 1). The amino acid candidates in particular have fulfilled many of the criteria for neurotransmitter function (Snyder & Young, 1975; Davidson, 1976; De Feudis, 1975). Although a neurotransmitter function for some of these substances may be difficult to envisage on account of their widespread distribution in the central nervous system and involvement in intermediary metabolism, functional compartmentation, as has been demonstrated for glutamate, may enable separation of their various functions. These amino acids may also act as modulators of neuronal function mediated by the recognized, more localized neurotransmitters (Barchas, Akil, Elliott, Holman & Watson, 1978; Schrier & Thompson, 1974).

Several of the putative amino acid neurotransmitters are metabolites of ammonia, which has featured prominently in the discussion of the pathogenesis of hepatic coma. These are glutamate and aspartate, which have excitatory action, and GABA, which is inhibitory. Changes in brain concentrations of glutamate and aspartate as well as in ammonia and glutamine have been found in studies of hepatic encephalopathy.

Many theories of hepatic coma have evolved around substances related to neurotransmission. In addition, several other substances have been implicated. Some of these hypotheses have been summarized in Table 2. As described in the General Discussion, several of the

TABLE 1

POSSIBLE CENTRAL NERVOUS SYSTEM NEUROREGULATORS (NEUROTRANSMITTERS AND NEUROMODULATORS)

	Compound	Precursor
Tertiary amine:	acetylcholine	choline, acetyl-CoA
Aromatic amines:	noradrenaline	tyrosine
	dopamine	
	octopamine	
	phenylethylamine	
	tyramine	
	histamine	histidine
	serotonin	tryptophan
	melatonin	
	tryptamine	
Amino acids:	aspartate	glucose, ammonia
	glutamate	
	γ-aminobutyric acid	
	glutamine	
	glycine	
	taurine	
Peptides:	oxytocin	
	vasopressin	
	angiotensin II	
	somatostatin	
	substance P	
	neurotensin	
	endorphins and enkephalins	
Lipids:	prostaglandins	
Nucleotide:	cyclic AMP	

TABLE 2

FACTORS IMPLICATED IN THE PATHOGENESIS OF HEPATIC ENCEPHALOPATHY

A. Related to neurotransmission

	Change in brain substance	Postulated result	Reference
	noradrenaline decreased	decreased excitation	Knell et al., 1974; Dodsworth et al., 1974
	serotonin increased	increased inhibition	Munro et al., 1975
	octopamine increased	displaces noradrenaline and dopamine	Fischer & Baldessarini, 1971
ammonia-related	GABA increased	increased inhibition	Goetchus & Webster, 1965
ammonia-related	α-ketoglutaramate increased	competes with glutamate	Vergara et al., 1974
ammonia-related	aspartate, glutamate decreased	decreased excitation	Hindfelt et al., 1977
ammonia-related	glutamine increased	displaces glutamate	Baldessarini & Yorke, 1974

B. Other factors

brain NH_4^+ increased	a	detoxification interferes with energy metabolism	Bessman & Bessman, 1955 Schenker et al., 1974
	b	competes with K^+ at neuronal membrane	Skou, 1962
plasma fatty acids and mercaptans increased		act synergistically with NH_4^+	Zieve et al., 1974
plasma methionine increased		--	Phear et al., 1956

neurochemical changes observed may be interdependent, resulting in a series of events which eventually lead to an imbalance in the neuroregulators, and consequent changes at the neuronal cell membrane.

The synthesis of the neurotransmitters acetycholine, serotonin and the catecholamines (noradrenaline and dopamine) is dependent on the availability of the respective precursors choline, tryptophan and tyrosine, which are originally obtained from the blood. In the case of serotonin and acetylcholine, the synthesis rate is in fact regulated by brain precursor concentrations, due to the low K_m of the rate-limiting enzyme for the substrate (Wurtman & Fernstrom, 1976). This means that serotonin synthesis may be controlled by tryptophan uptake which, in turn, is strongly influenced by the proportions of other plasma neutral amino acids competing for the same transport mechanism. Thus, it has been shown that serotonin synthesis rates are determined by plasma amino acid patterns via competition effects (Wurtman & Fernstrom, 1974). In addition, changing brain phenylalanine and tryptophan content may affect catecholamine synthesis by inhibiting the decarboxylation of dopa (Andrews, Patrick & Barchas, 1978). For these reasons much attention has been paid to the abnormalities in plasma amino acids both in absolute concentrations as well as distribution, found in the various forms of hepatic failure. It has been suggested that these abnormal patterns can promote the development of coma by causing abnormal uptake patterns of amino acids into the brain (Munro, Fernstrom & Wurtman, 1975; Soeters & Fischer, 1976).

When considering the effects of plasma amino acids on brain transport during hepatic encephalopathy, one assumption that has been

made is that the transport mechanisms are unchanged in hepatic failure. This may be far from the truth. A few studies have shown that the characteristics of the blood-brain barrier may be altered during hepatic failure (Livingstone, Hinchey & Goresky, 1974; Cremer, Lai & Sarna, 1977; Escourreau, James & Fischer, 1977). The finding that patients with liver disease have increased cerebral sensitivity to the effects of drugs and other insults such as infection, hypoxia and electrolyte disturbances (Schenker, Breen & Hoyumpa, 1974), could also suggest increased permeability of the blood-brain barrier. As the investigations of metabolite changes during hepatic encephalopathy for this thesis progressed, the results suggested that altered brain transport mechanisms could be involved. Therefore, studies of amino acid uptake in normal and encephalopathic conditions were included and are described in a separate section of the thesis.

The experimental models used to study hepatic encephalopathy have mostly involved surgery or the administration of chemicals. Chronic hepatic failure has been produced by performing a portacaval shunt in rats and dogs. The resulting changes in concentrations of amino acids and other metabolites seen in blood and brain, and of certain morphological changes in the brain, are similar to those present in patients with chronic liver disease. In addition, shunting of blood past the liver often occurs in these patients and appears to be a prerequisite for cerebral dysfunction (Sherlock, 1961). Therefore the portacaval shunted animal seems a suitable model for study of this condition.

In another model, rats with portacaval shunts were subjected to an acute ammonia challenge (Hindfelt, Plum & Duffy, 1977). These experiments were aimed at mimicking the situation in patients with

portal shunting who suffer from superimposed ammonia toxicity resulting from acute gastrointestinal bleeding or a sudden increase in protein intake.

Many studies have been made of metabolite changes in ammonia-induced coma in rats (Hindfelt & Siesjö, 197la,b; Hindfelt, 1972; Schenker, McCandless, Brophy & Lewis, 1967) but their relevance to hepatic coma can be questioned. Acute ammonia intoxication in experimental animals produces a hyperkinetic state (often with convulsions) with rapid turnover of metabolites and increased brain oxidative metabolism (Hawkins, Miller, Nielson & Veech, 1973). In addition, ammonia intoxication does not reproduce all the metabolic features of hepatic coma; for example brain serotonin, which is increased in hepatic coma, is unchanged in animals given large doses of ammonia (Walker, Speeg, Levinson & Schenker, 1971).

Experimental acute hepatic failure has been produced by total hepatectomy or by hepatic devascularization in dogs and rats. The two-stage ligation method, consisting of a portacaval shunt followed two days later by ligation of the hepatic artery, overcame many problems associated with other models such as total hepatectomy (death from bleeding diathesis) or simultaneous occlusion of the hepatic arterial and portal venous blood supply (death from shock) (Turcotte, Mattson & Child, 1967). In addition, the symptoms and biochemical abnormalities produced by this method more closely resembled changes seen in hepatic coma in man, than any of the previous models (Mattson, Iob, Sloan, Coon, Turcotte & Child, 1968).

Chemical methods of inducing acute hepatic failure have the advantage of involving much less trauma than surgical methods. The

selective hepatotoxin galactosamine has recently been used to cause acute hepatic necrosis and appears to have several advantages. Therefore this method as well as surgical methods were used to produce hepatic failure in the present studies.

This thesis describes investigations of altered neurotransmitter metabolism and related changes in brain and plasma metabolites, in chronic and acute hepatic encephalopathy. The effects of altering plasma amino acid patterns by intravenous infusion were examined. Finally, the possibility of an altered blood-brain barrier in hepatic failure was investigated by studying amino acid transport into the brain.

EXPERIMENTAL SECTION

A. SUMMARY

Metabolite changes were studied in two main groups of rats, namely those with chronic and acute hepatic failure. Chronic hepatic failure was studied four weeks after portacaval shunting while acute hepatic failure was produced either by portacaval shunt (pcs) followed by hepatic artery ligation (hal), or by intravenous injection of galactosamine-HCl (galN). These models are described in part C of this section. The findings in each condition were compared to the corresponding controls (sham-operated rats) and in some cases, normal rats. Intravenous feeding therapy was carried out in the chronic hepatic failure group.

Figure 1 summarizes the various conditions examined and indicates the time intervals between events.

Various clinical parameters and metabolites were studied. Clinical parameters included: behaviour, food consumption, and weight change; and during acute hepatic failure: body temperature and blood glucose. In the brain, concentrations of cyclic AMP, ammonia, glucose and various amino acids (Table 3) were measured. Plasma insulin, glucagon, glucose and amino acids were determined, and the relationships with brain metabolites investigated. In the acute hepatic failure series, brain water content was also measured to test for brain oedema.

In a separate study, the transport of tryptophan across the blood-brain barrier was investigated. Tryptophan uptake was measured at various concentrations and in the presence of inhibiting amino

FIGURE 1

SUMMARY OF SEQUENCE OF EVENTS IN EXPERIMENTS

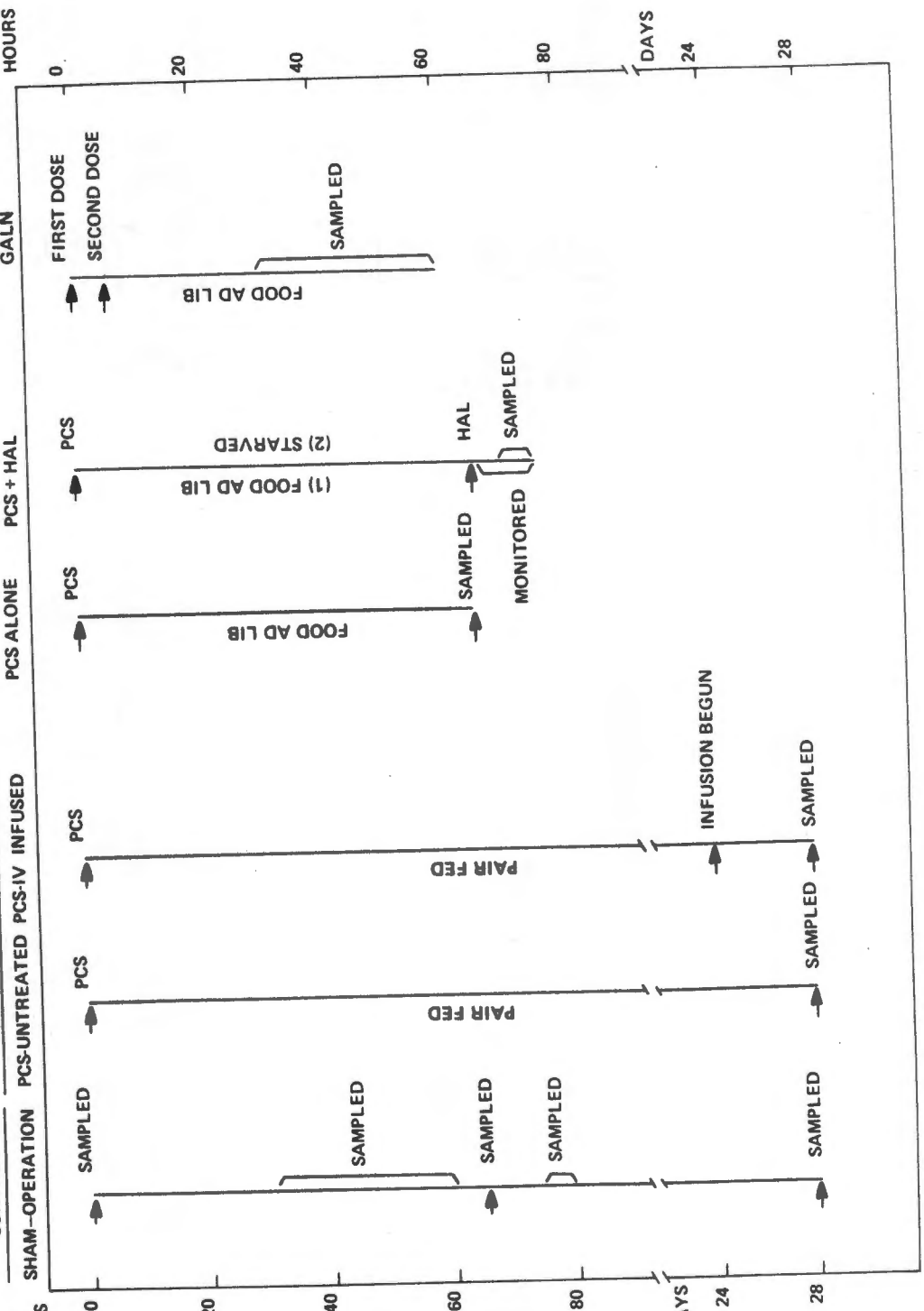


TABLE 3

AMINO ACIDS MEASURED IN BRAIN AND PLASMA

	Brain	Plasma
Essential:	tryptophan	tryptophan (non-albumin bound and total)
	tyrosine	tyrosine
	phenylalanine	phenylalanine
	methionine	methionine
	valine	valine
	isoleucine	isoleucine
	leucine	leucine
	threonine	threonine
Nonessential:	glycine	glycine
	alanine	alanine
	serine	serine
	aspartate	
	glutamate	
	glutamine	
	GABA	

acids, in both normal rats and rats with hepatic failure. The kinetic constants characterizing the system were calculated from the results and used to predict brain influx rates.

B. MATERIALS

RATS

All the rats used were adult Long-Evans males, weighing 300-350g and bred in the South African Mining Industries Research Centre for Heart Disease and Organ Transplantation at the University of Cape Town Medical School. The rats were housed under conditions of controlled temperature, humidity and lighting. The food consisted of commercial rat pellets (Epol, P. O. Box 497, Cape Town, South Africa) and contained: 20% protein, 4.3% fibre, 0.86% calcium, 0.72% phosphorus, 0.51% methionine, 1.20% lysine, and 7.0% fat, with added trace-elements and vitamins. Water and food were continuously available unless otherwise stated.

REAGENTS

The following enzymes and co-factors were obtained from Boehringer Mannheim GmbH Biochemica, Mannheim, West Germany: glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glutamate dehydrogenase (EC 1.4.1.3), glutamate-oxalacetate transaminase (EC 2.6.1.1), hexokinase (EC 2.7.1.1), malate dehydrogenase (EC 1.1.1.37), ADP, ATP, β-NAD^+, β-$NADP^+$, β-NADH, and β-NADPH. L-glutaminase (EC 3.5.1.2) was obtained from Sigma Chemical Corporation, St. Louis, U.S.A. [^{14}C]-Tryptophan and [^{3}H]-water, and all the reagents for the cyclic AMP assay, were obtained from the Radiochemical Centre, Amersham, England. [^{14}C]-n-Butanol was bought from New England Nuclear, 549 Albany St., Boston, Massachusetts, U.S.A. Scintillation cocktails Insta-gel and Dimilume-30, and tissue solubilizer Soluene-350 were bought from Packard Instrument Company Inc., 2200 Warrenville Road, Downers Grove, Illinois, U.S.A.

Dextrostix reagent strips were from Ames Company, Division of Miles Laboratory Ltd., Buckinghamshire, England. Heparin B.P. was obtained from The Boots Company (S.A.) (Pty) Ltd., 111 Garmor House, 121 Plein St., P. O. Box 1559 7A, Cape Town 8000, South Africa, and Trasylol (brand of aprotinin) from Bayer Pharmaceuticals (S.A.) (Pty) Ltd., P. O. Box 2953, 16 Milner Street, Metro Industria, Milnerton, Cape Town 8000, South Africa. Halothane was purchased from I.C.I. South Africa (Pharmaceuticals) Ltd., Leyds Streets, Braamfontein, Johannesburg 1500/1, South Africa. The intravenous solutions Normal Saline and Plasmalyte B were obtained from Baxter Laboratories, Inc., Deerfield, Illinois, U.S.A. Dextrose (50%) was obtained from Saphar Laboratories, P. O. Box 568, Durham Avenue, Salt River, Cape Town 8000, South Africa. The F080 amino acid mixture was obtained from McGaw Laboratories, Division of American Hospital Supply Corporation, Glendale, California, U.S.A. Oxygen was obtained from Afrox Ltd., P. O. Box 51, Jellico Avenue, Epping Industria, Cape Town 8000, South Africa. Substrates and other chemical reagents were of the highest available grade and were bought from Sigma Chemical Corporation or BDH Chemicals Ltd., Poole, England.

C. GENERAL PROCEDURES

Methods specific to particular sections are described in the relevant sections.

SAMPLING OF BLOOD AND BRAIN

For sampling, the rat was placed in position on the freeze-blowing apparatus (described below), and blood removed from the femoral artery into a heparinized syringe, immediately before sampling the brain. One ml of blood was immediately added to a pre-weighed tube containing two ml $HClO_4$ and extracted for glucose determination. One ml was transferred to a centrifuge tube containing 0.1 ml aprotinin, mixed thoroughly and centrifuged at 10 000 g_{max} for 15 minutes to obtain the plasma (for determination of glucagon). The remaining blood was centrifuged in the same way and all the plasma stored at -20° until analysis.

Brain was sampled by using the rapid freeze-blowing technique (Veech, Harris, Veloso & Veech, 1973). The apparatus used was made by Precision Medical Industries Inc., 105 Poquito Rd., Shalimar, Florida 32579, U.S.A. With this technique, two hollow stainless steel probes are driven into the cranial cavity of the rat held in a restraining cage, and pressurized gas entering one probe blows the supratentorial part of the brain through the other probe into a thin cavity between two aluminum discs precooled in liquid N_2. In this way brain samples of 0.5 to 1.0 g can be removed and frozen within 0.5 seconds. The frozen tissue was rapidly removed to a mortar containing liquid N_2, and transferred into precooled polyethylene tubes with screw caps for storage in liquid N_2 until extraction.

It has been shown that this method is superior to decapitation or whole-animal freezing for preventing post-mortem enzyme activity and therefore metabolite changes in brain samples (Nahorski & Rogers, 1973; Veech et al., 1973). In addition, no prior surgery or anaesthesia is required as with the method of exposing the brain and applying liquid nitrogen directly to the surface. Thus, the relationship between plasma and brain metabolites can be accurately correlated with the state of consciousness in vivo. One disadvantage is that specific regions of the brain cannot be analyzed. Since there is evidence that several amino acids and putative neurotransmitters, e.g. glutamine, glutamate and serotonin, are not homogenously distributed throughout the brain (Liebschutz, Airoldi, Brownstein, Chinn & Wurtman, 1977; Saavedra, 1977), results from total supratentorial brain samples may be less meaningful for these substrates or their precursors. In addition, the lower areas of the brain, in particular the cerebellum and lower midbrain, are not obtained.

For certain procedures the use of anaesthesia is unavoidable even when brain samples are obtained by freeze-blowing, for example, when catheters must be placed to obtain blood samples prior to sampling the brain.

In some experiments the rat was anaesthetized if necessary, the skull opened at room temperature, and the brain rapidly removed and placed in liquid nitrogen. In these experiments only those metabolites were measured which do not undergo changes during the sampling period, as compared to brain samples obtained by freeze-blowing. Blood samples were taken from the abdominal aorta in these cases.

In the galactosamine studies, samples of liver, kidney and spleen were taken from some animals and kept in isotonic formalin buffered to pH 7, for later histological evaluation.

EXTRACTION OF TISSUE

Frozen brain was ground to a fine powder using a pre-cooled pestle and mortar under intermittent irrigation with liquid N_2. The N_2 was allowed to evaporate and the dry brain powder rapidly weighed out into a tared pre-cooled polyethylene centrifuge tube. Six volumes of ice-cold 8% $HClO_4$ were added. After digesting for 15 minutes on ice and thorough mixing, the extract was centrifuged at 20 000 g_{max} for 15 minutes at 4°C, in a RC-5 Sorval centrifuge. The supernatant fluid was poured off and neutralized to pH 6-7 with cold, freshly prepared 15% KOH/15% K_2CO_3 (about 0.25 ml per ml of extract). More alkaline conditions were avoided as this may result in loss of NH_4^+ from the extract. The precipitate $KClO_4$ was removed by centrifuging as before and the supernatant used for determination of metabolites. Blood (one ml) was similarly extracted with two ml 8% $HClO_4$ and the extract neutralized as above. Weight of the brain powder and blood, and volumes of the extract before and after neutralization were noted for calculation of the dilution factor as follows:

$$\text{Dilution factor} = \frac{\text{weight tissue x 0.8 + } HClO_4}{\text{weight brain powder or weight blood}} \times \frac{\text{neutralized volume}}{\text{acidic volume}}$$

ASSAY PROCEDURES

1. Enzymatic Methods

The metabolites ammonia, aspartate, glutamine, glutamate and glucose were measured spectrophotometrically using nicotinamide adenine dinucleotides with appropriate cofactors and enzymes (Biebuyck, Lund & Krebs, 1972; Bergmeyer, 1974). These techniques involve the oxidation or reduction of the pyridine nucleotides

$$NAD^+ \rightleftharpoons NADH$$

and

$$NADP^+ \rightleftharpoons NADPH$$

The substrate to be determined is involved in a reaction with an associated change in the redox state of either NAD or NADP using NAD- or NADP-dependent dehydrogenases. Since the reduced form of these nucleotides absorbs light at a wavelength of 340 nm while the oxidized form shows no absorption between 300 and 400 nm, the reaction results in an increase or decrease in absorption of light at 340 nm which is measured spectrophotometrically. If conditions are chosen so that the reaction goes to completion in the desired direction, the observed change in absorption is proportional to the concentration of substrate in the cuvette. The equilibrium of the reaction can be influenced by variation of the conditions, such as pH, addition of excess substrate or enzyme, the presence of cofactors such as ADP, and the use of trapping agents, e.g. hydrazine hydrate, to remove products of the reaction.

Measurements were made in glass cuvettes with a light path length of one cm. The substrate concentration in the tissue extract was calculated from the change in absorption as follows:

$$\text{Concentration} = \frac{\Delta E}{6.3} \times \frac{\text{total volume in cuvette}}{\text{volume of extract used}} \ \mu\text{mol.ml}^{-1}$$

where ΔE is the change in absorbance in the sample cuvette after subtraction of the absorbance change in the reagent blank cuvette, and 6.3 is the extinction coefficient of NADH and NADPH at 340 nm in $\text{ml.}\mu\text{mol}^{-1}\text{.cm}^{-1}$. (Recent investigations [Bergmeyer, 1975] have established this more accurate value for the extinction coefficient; previously the value of 6.22 was used.) Multiplication of the concentration in the tissue extract by the dilution factor gave the substrate concentration in the brain per g wet weight tissue.

All determinations were carried out in duplicate, using a Zeiss PMQ III spectrophotometer with an automatic amplification unit. Standards were included in all assays. The reagent blank used was obtained by neutralizing 8% $HClO_4$ as in the tissue extraction procedure and using the same volume of supernatant as for the tissue samples.

Ammonia. NH_4^+ was measured using glutamate dehydrogenase (Kirsten, Gerez & Kirsten, 1963):

$$NH_4^+ + \alpha\text{-ketoglutarate} + NADH \longrightarrow \text{glutamate} + NAD^+ + H_2O$$

Excess α-ketoglutarate and enzyme shift the equilibrium to the right. ADP was added to overcome inhibition of glutamate dehydrogenase by ATP and GTP in the sample extract (Levitzki, 1970; Lund, 1971). Special precautions were taken to prevent ammonia contamination from the air in reagents and glassware. Only water that had been freshly double

distilled was used. (Distilled water from various sources was found to be seriously contaminated with ammonia, in one case the ammonia concentration was twice that of normal brain extract.) The cuvettes were soaked in chromic acid solution and only removed just before use. They were thoroughly rinsed with deionized water to remove all traces of acid and then rinsed several times with double distilled water. All reagents were freshly made up except the buffer and glutamate dehydrogenase solution which were stored at 4°C in tightly stoppered containers. Brain samples were always assayed for ammonia immediately after extraction, since freezing and thawing of the extract was found to lead to losses. Ammonia concentration in the blank cuvette was usually about 0.005 mM (mostly due to ammonia contamination of the KOH and K_2CO_3 used to make up the neutralizing solution).

Aspartate. Aspartate was determined in a two-step reaction (Bergmeyer, Bernt, Möllering & Pfleiderer, 1974):

(1) L-aspartate + α-ketoglutarate $\xrightarrow[\text{transaminase}]{\text{glutamate oxalacetate}}$ oxalacetate + glutamate

(2) Oxalacetate + NADH + H^+ $\xrightarrow[\text{dehydrogenase}]{\text{malate}}$ malate + NAD^+

The absorbance was measured five minutes after the addition of malate dehydrogenase, the glutamate oxalacetate transaminase added, and the second reading taken 10 to 20 minutes later.

Glucose. Glucose was determined in a two-step reaction (modified from Bergmeyer, Bernt, Schmidt & Stork, 1974):

(1) Glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-phosphate + ADP

(2) Glucose-6-phosphate + $NADP^+$ $\xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}}$ 6-phosphogluconate + NADPH + H^+

The first reading was taken 3 to 5 minutes after addition of glucose-6-phosphate dehydrogenase to allow for conversion of any glucose-6-phosphate present. Then the hexokinase was added and the second reading taken after a further five minutes.

Glutamine and glutamate. Glutamate was measured with glutamate dehydrogenase (modified from Bernt & Bergmeyer, 1974).

L-glutamate + H_2O + NAD^+ $\xrightarrow[\text{dehydrogenase}]{\text{glutamate}}$ α-ketoglutarate + NADH + NH_4^+

To determine glutamine, the glutamine in a portion of tissue extract was first converted to glutamate by incubation with glutaminase (Lund, 1974):

L-glutamine + H_2O $\xrightarrow{\text{glutaminase}}$ L-glutamate + NH_3

An aliquot of this reaction mixture was then assayed for glutamate, which represented the sum of glutamine and glutamate. The glutamate content of the extract was determined by assaying the extract directly and the glutamine determined by subtraction, i.e.

glutamine in extract = (glutamate present after hydrolysis) - (glutamate in extract).

2. Non-enzymatic Methods

Tryptophan. Free tryptophan was assayed in brain tissue extract and in plasma. In plasma, tryptophan is largely bound to albumin. (This is still regarded as free tryptophan however, since it is in equilibrium with the unbound tryptophan and does not form part of protein.) Both the total and unbound tryptophan concentrations in plasma were determined.

Separation of Unbound From Bound Tryptophan. Plasma ultrafiltrate was obtained by centrifuging 0.5 ml fresh plasma in Amicon 24-CF 50 Centriflo cones at 800 g_{max} for 15 minutes at room temperature (Knott & Curzon, 1972). (Speeds higher than 1000 g result in damage to the cone membrane.) These Centriflo cones have a 50 000 molecular weight cut-off point and therefore exclude albumin. A fraction of the filterable plasma components including the unbound tryptophan passes through the membrane. It has been suggested that this method may disturb the relationship between bound and unbound tryptophan by concentrating the albumin within the cone (Madras, Cohen, Messina, Munro & Wurtman, 1974). However, since unbound tryptophan is removed just as readily as the other filterable plasma components, the filtration procedure does not affect the binding equilibrium, even though an increase in the concentration of albumin and bound tryptophan occurs inside the cone. This can be seen from the following:

$$[\text{albumin}] + [\text{unbound tryp}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{bound tryp}]$$

The equilibrium constant

$$\frac{k_1}{k_{-1}} = K = \frac{[\text{bound tryp}]}{[\text{alb}][\text{unb tryp}]}$$

If half of the solute and half of the unbound tryptophan is removed, the concentration of albumin and bound tryptophan inside the cone is doubled, while there is no net change in unbound tryptophan concentration, i.e.

$$K = \frac{2[\text{bound}]}{2[\text{alb}] \times \frac{2[\text{unb}]}{2}} = \frac{[\text{bound}]}{[\text{alb}] \times [\text{unb}]}$$

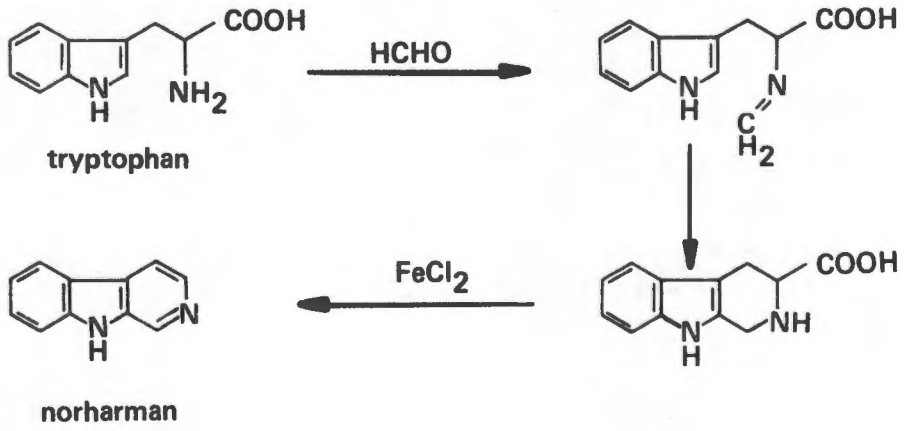
Thus there is no net change in K. This was confirmed for the present studies by experiments in which plasma was centrifuged for various lengths of time. No differences in unbound tryptophan concentration in the ultrafiltrate were obtained.

The alternative method of using equilibrium dialysis for 3.5 hours (Madras et al., 1974) has the disadvantages of being more tedious and possibly leading to artifical increases in the unbound tryptophan concentration (Hutson, Knott & Curzon, 1976).

Since the binding of tryptophan to albumin is pH sensitive (McMenamy & Oncley, 1958), blood was centrifuged immediately after collection before pH changes could occur due to loss of CO_2. Freezing of the plasma was found to lead to significant elevations in the levels of unbound tryptophan, therefore the ultrafiltrate was separated on the same day, at room temperature, before storing the ultrafiltrate and plasma at -20°C. The tryptophan was assayed within five days after collection of the sample. After use, the cones were rinsed with

distilled water, soaked overnight in 5% NaCl solution, rinsed with distilled water, and stored in 10% ethanol. They could be re-used up to 10 times.

Assay of Tryptophan. The free tryptophan is condensed with formaldehyde and oxidized by ferric chloride in acid conditions to form a highly fluorescent derivative, norharman. The norharman produced by the sample is measured spectrophotometrically and the concentration determined by comparison with standards.



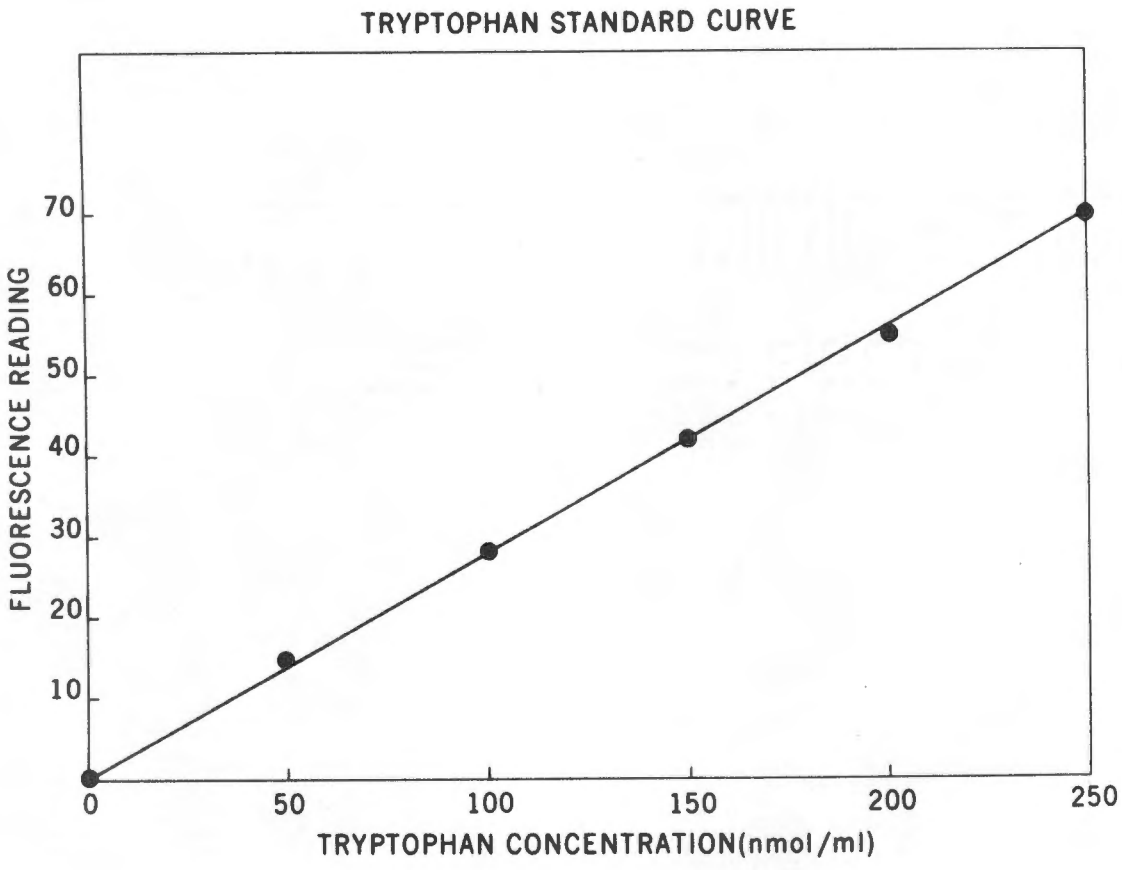
Of the naturally occurring tryptophan derivatives tested, only tryptamine produces any appreciable fluorescence with this method (Denckla & Dewey, 1967). However, since endogenous levels of tryptamine in rat brain are about 100 times lower than tryptophan (Eccleston, Aschcroft, Crawford & Loose, 1966) and the fluorescence produced by tryptamine is 10 times lower than that produced by the same concentration of tryptophan (Denckla & Dewey, 1967), the method can be considered to be highly specific for tryptophan.

In the original method described by Denckla and Dewey (1967) trichloroacetic acid was used, which was found to cause several problems.

Incubation with this acid at the high temperatures necessary for the reaction, resulted in decomposition to chloroform leading to blowing out of stoppers and loss of fluid, or the presence of an insoluble layer when screw-capped tubes were used. Therefore perchloric acid was used (Eccleston, 1973) and the incubation carried out in glass tubes with teflon-lined caps. A hot-air oven at 105°C was used in preference to a waterbath for incubation, since temperatures below 99°C seriously affect the yield (Denckla & Dewey, 1967) and higher temperatures were difficult to maintain in a waterbath.

The incubation mixture contained 1-8 nmoles tryptophan (from standards or samples), 1.5×10^{-4} M $FeCl_3$, 0.3% HCHO and 10% $HClO_4$ in a total volume of 3 ml. A range of six standards was included with each assay. Norharman standards were initially included to ensure that there was no loss during incubation. Plasma samples were allowed to stand for 10 minutes after addition of the $HClO_4$ to denature the albumin and release the bound tryptophan, and then centrifuged at 13 000 g_{max} for 10 minutes before adding the other reagents. Incubation was carried out at 105°C for three hours. The fluorescence was measured in a Perkin Elmer MPF 43A spectrofluorimeter at an excitation maximum of 373 nm and an emission maximum of 452 nm. The readings of the standards were used to calculate a calibration line by linear regression and the sample concentrations determined from this line. Over the range of standards used, the intensity of fluorescence increased linearly with increase in concentration of norharman or tryptophan, with a correlation coefficient of 0.998 or greater (Figure 2).

FIGURE 2



A range of tryptophan standards (0 to 250 nmol/ml in 0.1 M NH_4OH) was incubated with $FeCl_3$ and formaldehyde in perchloric acid as described in the text, and the resulting fluorescence measured at excitation maximum 373 nm and emission maximum 452 nm. The correlation coefficient r = 0.999.

Other amino acids. These were measured on a Beckman 120 C automatic amino acid analyzer. Plasma was deproteinized with 10% salicylsulphonic acid and 50 to 200 µl of the supernatant applied to the chromatographic columns. Neutralized brain extract (1 ml) was freeze-dried and reconstituted with 0.2 M citrate buffer pH 2.2 before amino acid analysis.

Cyclic AMP. Cyclic AMP was determined using a protein binding assay (Cyclic AMP Assay Kit, Amersham) according to the method of Tovey, Oldham & Whelan (1974) as modified from the original method of Gillman (1970). This assay is based on the competition between unlabeled cyclic AMP in the sample and a fixed quantity of added tritium-labeled compound for binding to a protein which has a high specificity and affinity for cyclic AMP. The amount of labeled protein-cyclic AMP complex formed is inversely related to the amount of unlabeled cyclic AMP present in the assay sample. Thus, measurement of the protein-bound radioactivity enables the amount of unlabeled cyclic AMP in the sample to be calculated. Separation of the protein-bound cyclic AMP from the unbound nucleotide is achieved by adsorption of the free nucleotide on to charcoal, followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabeled cyclic AMP is determined from a linear standard plot, obtained by plotting Co/Cx versus concentration of unlabeled standard cyclic AMP, where Co = counts bound in the absence of unlabeled compound, and Cx = counts bound in the presence of x picomoles of standard. A new standard line was determined each time the assay was performed, to check on the stability of the binding protein.

This method gave high precision and reproducibility. Initial unsuccessful attempts were made with the Assay Kit from Boehringer Mannheim GmbH in which millipore filters are used to adsorb the protein-cyclic AMP complex. However, only negligible radioactivity was retained on the filters; this finding was confirmed by other users of the same method.

Insulin and glucagon. Standard radioimmunoassay techniques were used to measure plasma insulin (Weinkove, Weinkove & Pimstone, 1974) and glucagon (Unger, Aquilar-Parada, Müller & Eisentraut, 1970). These assays were carried out by the Endocrine Laboratories of the Department of Medicine, University of Cape Town Medical School.

STATISTICAL ANALYSIS

Differences between means were tested for significance by using Student's t-test or the paired t-test where applicable (Snedecor & Cochran, 1967). Correlation coefficients were tested against the null hypothesis that $r = 0$. Calculations were carried out on a Tektronix calculator, provided with programs for statistical evaluations. Suitable programs were written for all other calculations.

D. DESCRIPTION OF ANIMAL MODELS

Although plasma samples may readily be obtained from patients with encephalopathy from acute or chronic liver disease, brain metabolites can obviously not be measured until after death. Such post-mortem measurements do not reflect the true in vivo situation. Cerebrospinal fluid metabolites have been measured during hepatic encephalopathy (Record, Chase, Curzon & Murray-Lyon, 1974; Lal, Young & Sourkes, 1975; Ono, Hutson, Dombro, Levi, Livingston & Zeppa, 1978) but there is some doubt about whether these are true indications of brain concentrations (Moir, Aschcroft, Crawford & Guldberg, 1970; Young, Etienne & Sourkes, 1976). Therefore, in order to study the cerebral effects of chronic and acute liver disease, suitable animal models are necessary.

Requirements for a suitable model of hepatic encephalopathy include:

(a) The method used should cause, as far as possible, selective liver damage.

(b) The liver damage produced should be reproducible.

(c) The time of onset of coma and death should be within a narrowly defined range.

(d) The clinical symptoms and brain and plasma metabolite changes should be similar to those observed in patients with hepatic failure.

(e) Ideally the liver damage and associated encephalopathy should be potentially reversible.

In general, surgical methods can be used to cause specific liver damage but usually involve considerable trauma and the damage caused is irreversible. Chemical methods are less traumatic and simpler to use, but may involve tissues other than liver and are often less predictable. Table 4 gives examples of various methods used.

The portacaval shunted dog and rat have been widely used to study chronic hepatic failure. The biochemical changes occurring after portacaval shunting in these animals are very similar to those found in patients with chronic hepatic encephalopathy. Experimental acute hepatic failure has been produced by surgical procedures such as hepatectomy or hepatic devascularization, i.e. portacaval shunt plus hepatic artery ligation, usually in two steps to reduce trauma. The response depends somewhat on the animal species used. This process, as with portacaval shunt alone, results in metabolite changes also seen in acute hepatic failure patients. The surgical trauma involved, however, remains a serious disadvantage of this method even when sham-operated controls are used. In a recent study the selective hepatotoxin galactosamine was used to induce liver necrosis and fulminant hepatic failure in rabbits (Blitzer et al., 1977). In these experiments, death was preceded by a period of clinically obvious coma, which is not always evident after portacaval shunt plus hepatic artery ligation. Therefore galactosamine-induced hepatic necrosis seemed to be a suitable alternative to surgically induced liver failure for examining acute hepatic encephalopathy.

In this section the various rat models used to study hepatic encephalopathy are described. Chronic hepatic failure was examined in rats four weeks after portacaval shunting, while acute hepatic failure

TABLE 4

STUDIES OF CHRONIC AND ACUTE HEPATIC FAILURE

1. CHRONIC HEPATIC FAILURE

Studies in man: Wu et al., 1955; Iber et al., 1957; Ning et al., 1967; Sherwin et al., 1974; Fischer et al., 1974; Maddrey et al., 1976; Ono et al., 1978.

Studies in animals with portacaval shunts:
Dogs: Fischer et al., 1975; Aguirre et al., 1974; Soeters et al., 1976b.
Rats: Curzon et al., 1975b; James et al., 1976; Cummings et al., 1976a; Hindfelt et al., 1977.

2. ACUTE HEPATIC FAILURE

Studies in man: Record et al., 1976; Knell et al., 1974; Fischer et al., 1976.

Studies in experimental animals:

Species	Method	References
rat	hepatectomy	Tyce et al., 1967
dog	hepatectomy	Tyce et al., 1967; McMenamy et al., 1965
rat	devascularization	Biebuyck et al., 1974 Cummings et al., 1976b Dodsworth et al., 1974
dog	devascularization	Mattson et al., 1968 Iob et al., 1970
pig	devascularization	Curzon et al., 1973b Buxton et al., 1974
pig	acetaminophen	Miller et al., 1976
rabbit	galactosamine	Blitzer et al., 1978
rat	galactosamine	Chirito et al., 1977
dog	2,4-dimethylnitrosamine	Aguirre et al., 1974
rat	carbon tetrachloride	Cacciatore et al., 1973

was produced either by portacaval shunt plus hepatic artery ligation or by galactosamine treatment.

1. CHRONIC HEPATIC FAILURE

PORTACAVAL SHUNT

The rats were anaesthetised and an end-to-side portacaval shunt constructed as described by Lee & Fisher (1961) and Lee, Arnot, Engelbrecht & Terblanche (1973). Using clean, but not sterile, surgical technique, the abdomen was opened and the intestines retracted laterally to expose the vena cava and portal vein. These vessels were occluded after mobilisation, using rubber shod hemostats. An elliptical opening was made in the vena cava, the portal vein was severed after ligation of the end proximal to the liver, and an anastomosis was performed with No. 7-0 braided silk attached to two three-eighths circle taper needles, using over-and-over stitches. The portal vein was completely occluded from 10 to 12 minutes. The patency of the shunt after four weeks was checked in four rats by performing a splenoportogram (performed by Dr. R. Hickman, Department of Surgery).

Sham-operated rats of the same weight served as controls. These rats were similarly anaesthetised and a laparotomy performed; the inferior vena cava and portal vein were isolated, simultaneously occluded for 12 minutes, and then released.

All rats recovered from the operation within 10 minutes.

POST-OPERATIVE HANDLING

The rats were housed for four weeks in individual metabolic cages which were constructed so that food consumption could be accurately

monitored. The powdered food was supplied at the end of a narrow tunnel so that no food could be carried back into the main cage. Any food dropped in the tunnel fell through a grid into a trough, enabling all uneaten food to be weighed.

Each sham-operated rat was paired with a shunted rat and given the amount of food consumed by the shunted rat during the previous 24 hours. The rats were weighed twice weekly. At the end of the four week period the rats were anaesthetised with halothane (1.5% v/v in oxygen) and blood and brain samples obtained. The blood was taken from the abdominal aorta and the brain removed rapidly at room temperature. The samples were then treated as described under General Procedures.

WEIGHT LOSS AND BEHAVIOUR

The shunted rats lost about 10% of their pre-operative weight during the first 2-3 days after the operation. They continued to eat less food than normal rats and did not regain their pre-operative weight although weight loss became very slight or ceased after the first week. These findings were subsequently confirmed by others (Rossouw, Labadarios, Vinik & De Villiers, 1978). At the time of sampling there was no difference in mean percentage weight loss between the shunted rats and pairfed controls:

portacaval shunt	-11.3 ± 2.3 (S.E.M.) (8 rats)
control	-11.4 ± 1.4 (S.E.M.) (8 rats)

Behaviourally the two groups of rats could not be differentiated.

2. ACUTE HEPATIC FAILURE: SURGICALLY INDUCED

The procedure of using a two-stage interruption of hepatic blood supply to induce acute hepatic coma was first described by Rappaport, McDonald & Barowy (1953) using dogs. The animals were subjected to a side-to-side portacaval shunt and the hepatic artery was ligated 24-48 hours later. Later work, using an end-to-side anastomosis followed by ligation of the hepatic artery one to ten days later, showed that coma only developed if the ligation occurred within 72 hours after shunting (Giges, Dein, Sborov, Seligson & Howard, 1953; Turcotte et al., 1967). The dogs became comatose 16-24 hours after ligation. The degree of hepatic insufficiency and number of animals dying from hepatic coma could be altered by varying the time interval between the two stages. With a 24 hour interval nearly 100% mortality from coma resulted. With longer time intervals the percentage of animals in coma decreased, possibly due to a compensatory increase in the collateral arterial blood supply occurring after construction of the portacaval shunt (Mattson & Turcotte, 1969).

The surgical mortality rate obtained with the two-stage ligation method was much lower than that obtained with other methods such as total hepatectomy or simultaneous occlusion of the hepatic arterial and portal venous blood supply (Mattson & Turcotte, 1969). In addition, many of the symptoms and biochemical abnormalities produced closely resembled changes seen in hepatic coma in man.

Similar results were obtained in pigs after portacaval anastomosis followed immediately by hepatic artery ligation (Curzon et al., 1973b; Buxton et al., 1974; Opolon, Lavallard, Huguet, Bidallier, Granger, Gallot & Bloch, 1976). After the operation, liver function was grossly

impaired, movements became uncoordinated and coma ensued. Most animals died 5½ to 8½ hours after the operation (Curzon et al., 1973b).

In rats, acute hepatic failure has been induced by performing an end-to-side portacaval anastomosis followed 24 to 48 hours later by hepatic artery ligation (Fischer & Baldessarini, 1971; Dodsworth et al., 1974; Funovics, Cummings, Shuman, James & Fischer, 1975). In these studies the rats appeared normal for about 4 hours and then became increasingly somnolent and comatose, unresponsive to all but severe stimuli. They died in coma 6 to 12 hours after hepatic artery ligation. A similar method was used for the present studies.

PROCEDURE

The rats were subjected to a portacaval shunt or sham-operation as described for chronic hepatic failure. Postoperatively the rats were housed three to a cage with free access to food and water until sampling or hepatic artery ligation 65 hours after portacaval shunt. This time period allowed recovery from the first operation. The hepatic artery was ligated (Dodsworth et al., 1974) using halothane as anaesthetic (1.5% v/v in O_2). At the same time catheters were inserted into the femoral vein for the administration of glucose (17 g needle, 8 inch Deseret Intracath), and femoral artery for the removal of blood samples (19 g needle, 8 inch Davol catheter). Control rats were treated similarly except that the hepatic artery was not ligated. This procedure was completed within 20 minutes. Following hepatic artery ligation or sham-operation, the rats were placed in restraining cages and kept in a heated room to maintain rectal temperature at 37°C. Blood glucose levels were measured half hourly using Dextrostix and

maintained between 4.5 and 6.5 mM by giving 50% dextrose, diluted with normal saline, into the femoral vein. Blood glucose concentrations started to decrease soon after hepatic artery ligation and unless additional glucose was given, decreased to below 2.5 mM. To maintain normal glucose concentrations, 50-100 mg given half-hourly was usually sufficient. In the later stages there was often a more rapid decrease in blood glucose. Control rats did not require supplementary glucose. At the time of brain sampling there was no difference in blood glucose concentrations between the two groups. The mean concentrations ± S.E.M. in μmol/ml were:

hepatic devascularization	4.91 ± 0.96 (8 rats)
control	5.76 ± 0.22 (7 rats)

The rats were sampled 4 to 7 hours after hepatic artery ligation when they were unresponsive to all but severe stimuli. One group of rats was sampled 65 hours after portacaval shunt, i.e. without ligation of the hepatic artery. Control rats were sampled at the same time after surgery as the corresponding shunted rats. The brains were removed by freeze-blowing or at room temperature and stored in liquid nitrogen until extraction.

BODY WEIGHT LOSS

During the two days following the shunting operation both the shunted rats and sham-operated controls lost about 10% of their preoperative weight:

portacaval shunt	-9.8 ± 0.7 (S.E.M.) (25 rats)
control	-10.4 ± 1.0 (S.E.M.) (14 rats)

BEHAVIOUR

No change in behaviour was noted after portacaval shunt alone, or in the control rats, during the entire period of the experiment. After ligation of the hepatic artery, the rats behaved normally for the first three to four hours. Thereafter they became increasingly less active and unresponsive to stimuli. They were capable of being aroused but immediately returned to the inactive state. The EEG of several animals was monitored using a Cerebral Functions Monitor (Devices Instruments Ltd., 13-15 Broadwater Rd., Welwyn Garden City, Hertfordshire, England) and typical changes reported in patients (Kennedy, Parbhoo, MacGillivray & Sherlock, 1973) were not seen until immediately before death (6 to 9 hours after hepatic artery ligation) despite changes in behaviour. In order to avoid the secondary alterations in cerebral metabolism induced by the terminal events of hypoxia and decreased cerebral blood flow, brain samples were taken during the period of altered behaviour described above.

BRAIN OEDEMA

The brain water content was determined in a separate series of rats. Portions of the brain were rapidly weighed and then heated in a hot-air oven until there was no further loss of weight. The % water was determined from the total weight loss. No evidence was found of brain oedema in the rats with acute hepatic failure as indicated by the

brain water content. The mean % water content in the two groups of rats was:

hepatic devascularization	76.82 ± 0.61 (S.E.M.)	(5 rats)
control	76.78 ± 1.2 (S.E.M.)	(5 rats)

Oedema has been reported in rats 4-5 weeks after portacaval shunt (Cavanagh, 1974) as well as in patients dying of massive hepatic necrosis (Ware et al., 1971; Bernstein, 1976), while others found no increase in brain water content (Kindt, Brock, Altenau & Pöll, 1977). In view of these findings, the results suggest that cerebral oedema is a sequel to and not a causative factor in the development of acute hepatic encephalopathy.

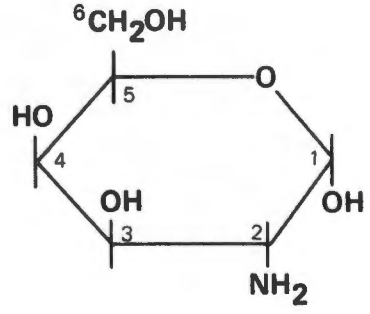
3. ACUTE HEPATIC FAILURE: GALACTOSAMINE-INDUCED

The hepatotoxin D-galactosamine (galN) has been shown to produce morphological and biochemical changes in the liver very similar to those found during human viral hepatitis (Keppler & Decker, 1969; Keppler, Lesch, Reutter & Decker, 1968; Reutter, Bauer, Kreisel & Lesch, 1971). It has been used to study metabolism and regulatory mechanisms of the liver cell. Because of its specificity and ability to cause massive liver necrosis, its use was recently suggested for producing a model of fulminant hepatic failure (Blitzer et al., 1977). In this study rabbits were injected intravenously with 4.25 mmoles/kg of galactosamine-HCl. Of 22 rabbits, 16 died between 22 and 41 hours after injection, 4 died between 48 and 89 hours, and 2 survived. In non-survivors a period of coma lasting 2.6 ± 4 (S.E.M.) hours preceded

death. These results suggested that galN might be useful in providing an alternative model to the surgical one for studying acute hepatic coma. Therefore, the experiments described in this section were carried out. The main objectives were to establish a predictable rat model of acute hepatic coma, involving minimal surgical trauma, and to compare the metabolic changes with those found during surgically induced acute hepatic failure.

BIOCHEMISTRY

GalN is the 2-amino substituted form of galactose:



2-amino-2-deoxy-D-galactose

GalN cannot be detected as the free sugar in untreated animals, but is found as a constituent of glycoproteins and polysaccharides, e.g., hyaluronic acid, heparin and cerebral gangliosides, as the N-acetyl conjugate with other hexoses. When administered intraperitoneally or intravenously, galN is taken up by the liver and enters the galactose metabolism pathway. It is rapidly phosphorylated by liver galacto-kinase:

1. GalN + ATP $\xrightleftharpoons{\text{galactokinase}}$ GalN-1-phosphate + ADP

2. GalN-1-phosphate + UDPG $\rightleftharpoons$ UDP-galN + glucose-1-phosphate

The second reaction is catalyzed by the enzyme galactose-1-phosphate: UDPG-uridylyltransferase which is nonspecific but has a low affinity for the unusual substrate. This low affinity and the limited supply of UDPG (derived from UTP) favour the accumulation of galN-1-phosphate. GalN-phosphate inhibits the enzyme UDPG-pyrophosphorylase which catalyzes the formation of UDPG from UTP:

$$\text{UTP} + \alpha\text{-D-glucose-1-phosphate} \rightleftharpoons \text{UDPG} + PP_i$$

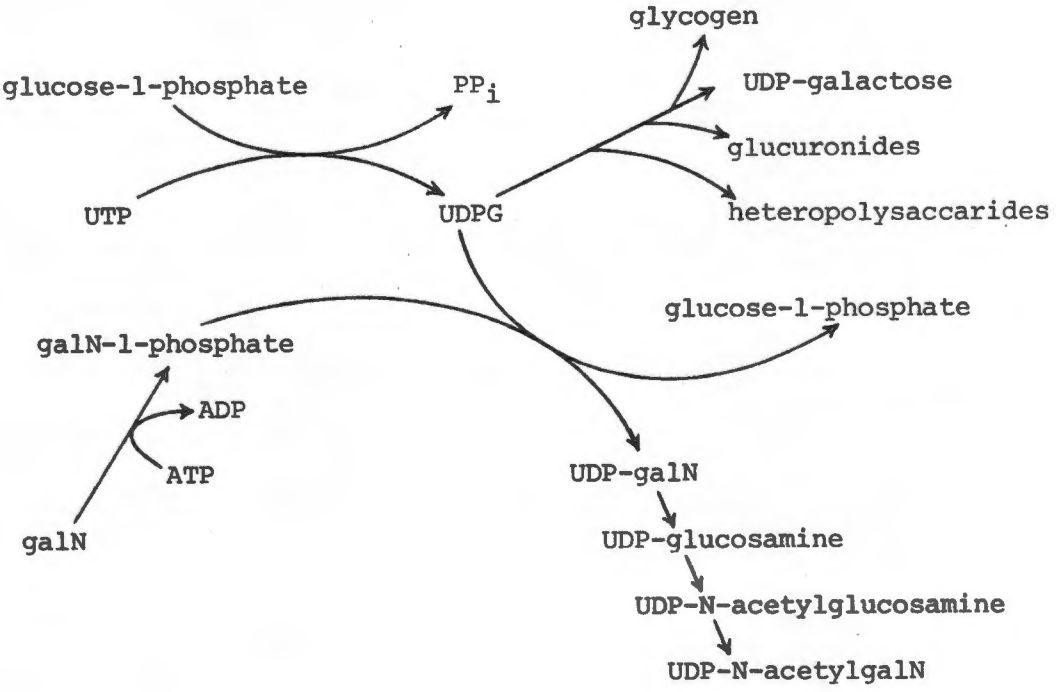
Therefore, as a result of galN-phosphate synthesis and accumulation, UDPG is depleted and its synthesis inhibited, further increasing the accumulation of galN-phosphate. UDP-galN is converted to UDP-glucosamine by UDP-galactose-4'-epimerase:

3. UDP-galN ⟶ UDP-glucosamine

UDP-glucosamine apparently does not serve as a uridylate donor (as does UDP-glucose which would be formed from galactose) so that UDP-hexosamine formation acts as a trap mechanism for uridylate. GalN-induced trapping of uridylate exceeds the compensatory capacity of uridylate biosynthesis about fourfold in the adult rat liver. Hence galN metabolism is associated with a rapid decrease in the concentrations of UDPG, UDP-galactose, UTP, UDP, UMP and UDP-glucuronate. Hepatic UTP levels drop to 20% of normal 15 minutes after an intraperitoneal injection of galN and to 7% after 30 minutes (Decker & Keppler, 1972). Control levels are reached again about 20 hours after a single dose. Only if concentrations of UTP or UDP-glucose remain below a critical level of about 25-30% of

normal for several hours will signs of cell damage appear later (Decker, Keppler, Rudigier & Domsche, 1971).

Further metabolites UDP-N-acetyl-glucosamine and UDP-N-acetyl-galN accumulate after galN-administration but these substances themselves do not cause hepatic injury (Decker & Keppler, 1972).



Uridylate deficiency results in depression of uracil nucleotide-dependent biosynthesis of macromolecules such as nucleic acids, glycoproteins, glucolipids in membranes, and glycogen, which is almost completely exhausted in liver cells of treated animals. Organelle injury in viable cells results, leading to liver necrosis. Necrosis of single liver cells is evident at 6 hours after a single dose of galN (Koff,

Gordon & Sabesin, 1971). After 26 hours all histologic signs of a spotty necrotic hepatitis are present, with an increase in hepatocellular fat when higher doses are used (Koff et al., 1971). The histologic changes are more severe 48 hours after injection and resemble those seen in acute liver necrosis (Keppler et al., 1968). In surviving animals the liver morphology returns to normal within 7 to 12 days. Repeated injections of galN can lead to hepatic cirrhosis in 6 months (Lesch, Keppler, Reutter, Rudigier, Oehlert & Decker, 1970).

No damage to other organs has been observed in rats except for frequent haemorrhages in gut and stomach, probably due to prothrombin deficiency (Keppler et al., 1968).

PROCEDURE

The rats were anaesthetised with halothane in oxygen and the femoral vein cannulated with a Bardi-Cath catheter (17 gauge needle). The galN solution was injected very slowly to prevent osmotic shock. Some rats were given a second dose 3-12 hours later and these were placed in restraining cages and the femoral line kept open by a very slow 0.9% sodium chloride infusion (0.6 ml/hour) until the second dose had been given. Control rats were given an equal volume of 0.9% sodium chloride solution. After dosing, the catheter was removed and the animals housed three to a cage with free access to food and water.

The dose of galN given ranged from 1 to 2½ g/kg body weight in a volume of 1-2 ml. GalN (as the hydrochloride salt) was dissolved in 0.1 M NaOH solution and brought to pH 7.4 with 1.0 M NaOH solution. The solution was injected at room temperature.

Metabolite concentrations were determined in samples obtained from rats given 2 g/kg galN in two doses three to six hours apart. These rats were divided into two groups based on their behaviour at the time of sampling, i.e. a "precoma" and a "coma" group (defined below). The results from the two groups were combined except where stated otherwise. At the time of sampling (some animals required light anaesthesia with halothane) four ml blood was sampled from the abdominal aorta through a large needle. The skull was then opened and the brain rapidly removed and placed in liquid nitrogen. The blood and brain samples were treated as described under General Procedures. Samples of liver, kidney and spleen were taken from several animals and kept in formalin buffered to pH 7 for routine histological evaluation.

CLINICAL FEATURES

The rats recovered rapidly after dosing and remained normally active for the first 24 hours, except for a loss in weight of about 10 g. Thereafter they showed signs of increasing drowsiness; they developed staring fur, their food intake decreased, and they continued to lose weight. They became progressively less responsive to stimuli until they lapsed into a clearly distinguishable coma 30 to 60 hours after the first galN dose. Death occurred 1-2 hours later. (For sampling purposes, coma was defined as the completely unresponsive condition. Rats responsive to severe stimuli only, and expected to become comatose during the next few hours, were defined as precomatose.) There was a large variation in time of response which was partly related to the amount of galN given and the weight of the rat. The most consistent

response was obtained by giving 2 g/kg in 2 doses 3-6 hours apart to rats weighing 340 to 350 g. (It has been shown that for liver cell damage to occur, the hepatic uridylate concentrations must be depressed below 30% of normal for several hours [Decker & Keppler, 1974]. This could be the reason for the improved response when divided doses were given.) Of 29 rats given 2 g/kg in 2 doses 3-6 hours apart, 16 rats (55%) became comatose between 30 and 48 hours after the first injection, 11 rats (38%) between 48 and 60 hours, and 2 rats (7%) recovered completely from the treatment. Younger rats were more resistant to the drug probably because of higher rates of hepatic uridylate biosynthesis (Keppler & Decker, 1971). For example, when 6 rats weighing 245-255 g were given 2 g/kg galN, only one became comatose. The other 5 rats recovered completely although all lost about 15 g weight during the first 48 hours and 3 showed signs of drowsiness.

HISTOLOGY

At the time of sampling the livers appeared pale and faintly speckled. Light microscopic examination showed massive cell necrosis, inflammation, haemorrhage, and fatty infiltration, with no evidence of mitochondrial activity. These findings agree with those previously reported (Keppler et al., 1968; Koff et al., 1971). No abnormality was seen in kidney or spleen tissue. In some rats there was evidence of haemorrhages in the gut and from the nose (probably due to prothrombin deficiencies).

4. DISCUSSION

Chronic hepatic failure induced by portacaval shunting results in many biochemical changes also seen in human chronic hepatic encephalopathy. Another manifestation of both the experimental and the human condition is an increase in brain astrocytes showing the Alzheimer Type II change. For these reasons the portacaval-shunted rat has been widely studied as a model of chronic hepatic encephalopathy. Whether it truly represents the syndrome of confusion, stupor and delirium observed in humans, is uncertain, since, certainly in this study, there were no obvious behavioural abnormalities in the shunted rats. Sophisticated behavioural monitoring may be the only way to answer this question.

The surgical model of inducing acute hepatic failure similarly results in metabolite changes, including hypoglycaemia, also seen in acute hepatic failure patients. In addition these rats showed clear signs of brain dysfunction, although a completely unresponsive state was not reached in most cases. The surgical trauma involved however remains a serious disadvantage of this method even when sham-operated controls are used. The use of galactosamine therefore appeared to be promising. The surgical manipulation was minimal in comparison to portacaval shunting plus hepatic artery ligation, and since the method was a completely different one it was interesting to see if similar biochemical and behavioural results would be obtained. The biochemical alterations were in fact very similar in the two models (see next two sections) indicating that these changes were due to liver failure and not the result of either surgical manipulation or the drug treatment.

Another advantage of using galactosamine was that the rats lapsed into a distinguishable coma before death. A disadvantage of the method was the wide variation in time of response which prevented adequate monitoring of physiological parameters such as temperature and blood glucose in the terminal stages. Intermittent monitoring of the EEG by means of implanted electrodes may be one way of following the mental state and approaching coma in this model.

RESULTS SECTION I

NEUROTRANSMITTERS: RELATED TO AMMONIA METABOLISM

Ammonium ion has long been a favourite candidate as the primary aetiologic agent in the development of hepatic encephalopathy. During liver disease with encephalopathy, ammonia concentrations are increased in the blood and cerebrospinal fluid (McDermott, 1958; Caesar, 1962; Plum & Hindfelt, 1976). Patients with liver damage are especially sensitive to the cerebral toxic effects of ammonia and often show intolerance of protein ingestion (Breen & Schenker, 1972). This evidence favoured the role of ammonia and its metabolism in the pathogenesis of hepatic coma. However, it is now generally believed that ammonia, although toxic to the brain, is not entirely or even primarily responsible for the development of hepatic coma. The correlation between ammonia blood levels and grade of hepatic encephalopathy is often poor and blood ammonia concentrations may be normal during severe encephalopathy (Phear, Sherlock & Summerskill, 1955; Summerskill, Wolfe & Davidson, 1957).

Brain metabolism of ammonia occurs by the formation of glutamate and glutamine according to the reactions:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH \underset{\text{dehydrogenase}}{\overset{\text{glutamate}}{\rightleftharpoons}} \text{glutamate} + NAD^+$$

$$\text{glutamate} + NH_4^+ + ATP \xrightarrow[\text{synthetase}]{\text{glutamine}} \text{glutamine} + ADP + P_i$$

A number of basic cerebral mechanisms were suggested for ammonia toxicity. The first and most widely accepted hypothesis was that NH_4^+ impaired brain energy metabolism and thus led to a decrease in the

available high energy phosphates (Bessman & Bessman, 1955; Breen & Schenker, 1974). Increased glutamine synthesis due to elevated NH_4^+ levels may hypothetically interfere with brain energy metabolism by various mechanisms:

a. α-Ketoglutarate may be diverted from the Krebs cycle at such a rate that substrate depletion occurs within the cycle, resulting in a slower rate of oxidation.

b. NADH availability for oxidation in the electron transport chain may be decreased, thereby lowering oxygen consumption and energy production.

c. The amidation of glutamate to glutamine is ATP-consuming, increasing the energy demands of the tissue.

The hypothesis that NH_4^+ causes encephalopathy by interference with brain energy metabolism as outlined above is no longer tenable for various reasons: it has been established that there is no shortage of high energy intermediates in experimental hepatic coma (Biebuyck et al., 1974); no distinctive abnormality of glucose oxidative metabolism could be found in dogs in hepatic coma after hepatectomy (Zieve et al., 1975); and NH_4^+ intoxication does not lead to depletion of α-ketoglutarate or cause primary energy failure (Hindfelt et al., 1977; Hindfelt & Siesjö, 1971a; Hawkins et al., 1973). In addition, synthesis of glutamine appears to use a small pool of glutamate that is rapidly turning over rather than the larger pool derived from α-ketoglutarate in the Krebs cycle (Berl & Clarke, 1969). Regional brain depletion of ATP and creatine phosphate has been reported (Schenker et al., 1967; Zieve, Zieve & Gilsdorf, 1972) and implicated in the development of coma (Schenker et al., 1974). However, the interpretation of these results

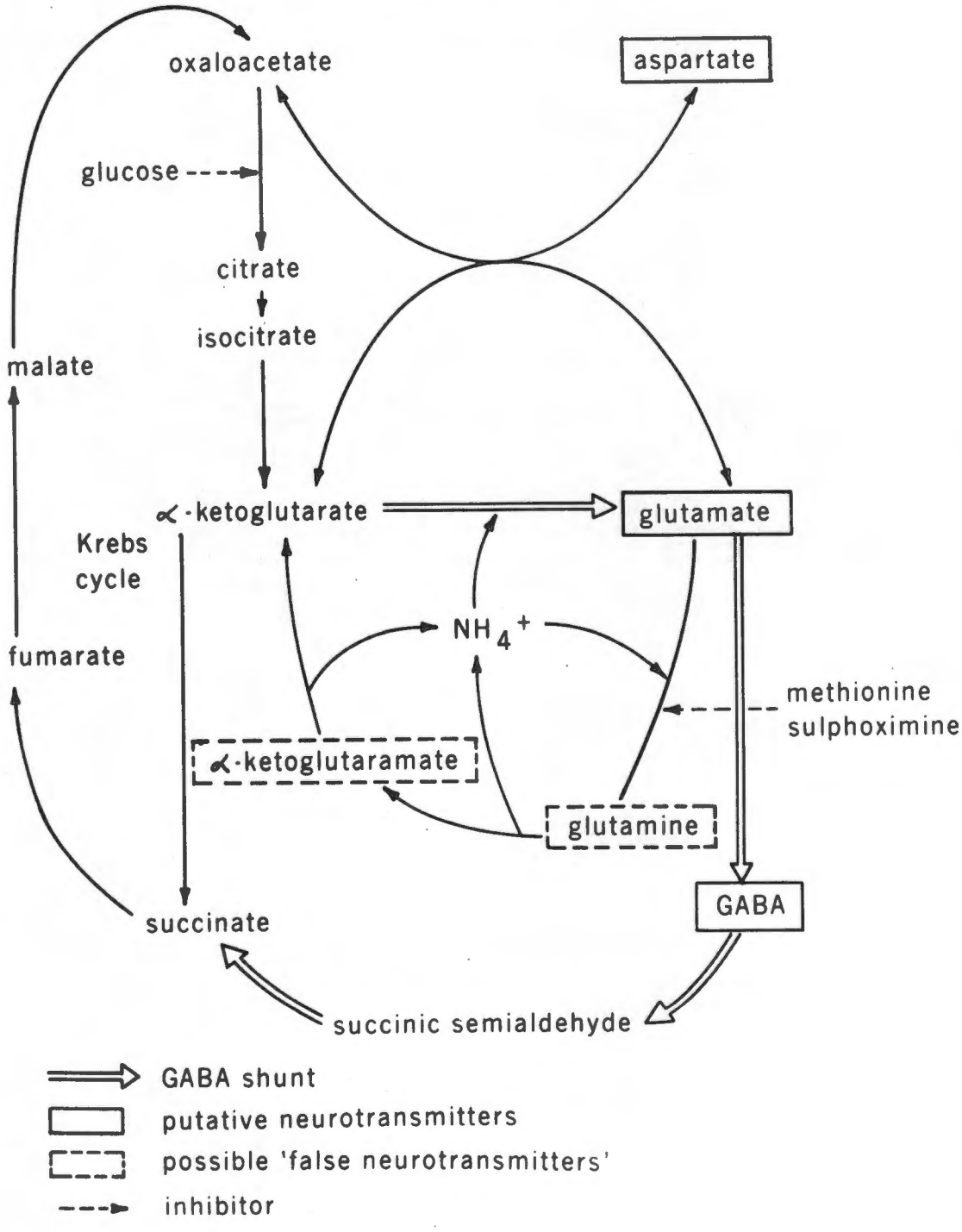
has been complicated by the various methods used for sampling brain tissue and investigating brain high-energy phosphates.

Other possibly toxic effects of ammonia have been suggested such as inhibition of the synthesis of acetylcholine by inhibition of pyruvate decarboxylase and depletion of ATP, thus diminishing acetyl CoA. However, in ammonia-intoxicated animals no changes in these metabolites have been noted (Schenker et al., 1967). A third suggestion is that the ammonium ion exerts a direct toxic effect on the neuronal membrane by competition with K^+ (Skou, 1962). In vitro ammonia decreases ATP-ase activity and could therefore disturb energy-dependent Na-K flux across the membrane (Skou, 1962). This possibility is so far untested.

During hepatic failure, changes have also been found in several amino acids associated with the metabolism of ammonia, specifically glutamine, glutamate, aspartate, γ-aminobutyrate (GABA) and α-ketoglutaramate (see Figure 3 for interrelationships). Glutamine is increased in plasma, cerebrospinal fluid and brain during hepatic encephalopathy in man and experimental animals (Hourani, Hamlin & Reynolds, 1971; Biebuyck et al., 1974; Record, Buxton, Chase, Curzon, Murray-Lyon & Williams, 1976). In contrast to ammonia, a fairly good correlation between glutamine and the degree of encephalopathy has been reported (Hourani et al., 1971). Glutamate and aspartate were decreased during acute hepatic failure in dogs (Mattson et al., 1968) and rats (Biebuyck et al., 1974). Glutamate was decreased three weeks after portacaval shunting in the rat (Hindfelt et al., 1977). Similar changes have been found in ammonia-induced coma in rats (Hindfelt & Siesjö, 1971a; Schenker et al., 1967). In fulminant hepatic failure

FIGURE 3

INTERRELATIONSHIPS BETWEEN AMMONIA AND THE GLUCOSE-DERIVED AMINO ACIDS



in man aspartate was decreased in the brains of some patients (Record et al., 1976).

These changes are of great interest in view of the postulated neurotransmitter roles of several of the amino acids associated with the metabolism of ammonia, namely GABA, glutamate and aspartate (Curtis & Johnston, 1970; Hebb, 1970; Snyder, Young, Bennett & Mulder, 1973; De Feudis, 1975; Davidson, 1976). These amino acids, as well as glycine and taurine, are unique among amino acids in that they all have specialized high-affinity uptake systems in mammalian brain in addition to being transported by relatively nonspecific low-affinity transport systems as are the other amino acids (Iversen, 1971; Logan & Snyder, 1972). Since similar high-affinity systems are found for the neurotransmitter amines, this is strongly suggestive that these systems may play a role in terminating the actions of the amino acids after their synaptic release, as with the catecholamines at adrenergic synapses.

α-Ketoglutaramate is derived from glutamine by the removal of an amine group (see Figure 3). Its concentration is increased in the cerebrospinal fluid of patients with encephalopathy (Vergara, Plum & Duffy, 1974) and the suggestion was made that it could exert a toxic effect by competition with glutamate for post-synaptic receptors. The finding that methionine sulphoximine, which inhibits glutamine synthetase (see Figure 3), could reduce ammonia toxicity in mice (Warren & Schenker, 1964), seemed to fit in with this suggestion. However, when α-ketoglutaramate was perfused into cerebral lateral ventricles of rats, coma was not produced, although depressed nocturnal locomotor activity resulted (Duffy, Vergara & Plum, 1974). More recently it was found that α-ketoglutaramate neither alters cerebral cortical synaptic

activity nor impairs the metabolism of cerebral tissue slices and is therefore probably a nontoxic byproduct (Plum, 1978).

Attention was recently focused on a completely different substance, cyclic AMP, which seems to be involved in neurotransmission in various ways, and which was found to be greatly increased during anaesthesia (Biebuyck, Dedrick & Scherer, 1975).

The changes in brain concentrations of ammonia and the ammonia-related putative neurotransmitter amino acids as well as cyclic AMP, observed during hepatic failure, are discussed in this section.

RESULTS

Table 5 shows the concentrations of brain NH_4^+ and its metabolites in rats four weeks after portacaval shunt and in controls. There were significant increases in NH_4^+ (2.5-fold) and glutamine (nearly 3-fold) after portacaval shunt. A small but significant decrease was found in aspartate. No changes were found in concentrations of GABA or glutamate. In an earlier study of the portacaval shunted rat similar results were found; however, in this study there were decreases in glutamate but not aspartate (Hindfelt et al., 1977). On the other hand, aspartate was decreased during ammonia-induced precoma and coma in rats with portacaval shunts (Hindfelt et al., 1977).

The concentrations of brain ammonia and metabolites after portacaval shunt alone, after hepatic devascularization, and in control rats, are presented in Table 6, while results from the galN study are shown in Table 7. (Since there was no difference in these metabolites between the coma and precoma groups, the two groups were combined for this table.)

TABLE 5

CHANGES IN BRAIN AMMONIA AND ITS METABOLITES DURING CHRONIC HEPATIC FAILURE

All rats were sampled four weeks after portacaval shunt (pcs) or sham-operation (control). The results are expressed in μmol/g wet weight tissue as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from control are indicated by *p<0.01, **p<0.001.

	Control	Pcs
NH_4^+	0.40 ± 0.04 (7)	0.97 ± 0.10 (9)**
Glutamine	5.85 ± 0.30 (11)	15.9 ± 1.0 (13)**
Glutamate	10.5 ± 0.4 (11)	10.1 ± 0.4 (13)
Aspartate	2.50 ± 0.08 (8)	2.16 ± 0.07 (4)*
GABA	2.6 ± 0.1 (11)	2.3 ± 0.1 (10)

TABLE 6

BRAIN AMMONIA AND METABOLITES AFTER PORTACAVAL SHUNT AND PORTACAVAL SHUNT PLUS HEPATIC ARTERY LIGATION

Brain samples were taken from two groups of rats; following portacaval shunt (pcs), and following portacaval shunt and hepatic artery ligation (hepatic devascularization). Each group was compared to the corresponding controls. (See Experimental Section for further details.) The results are expressed in μmol/g wet weight tissue as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from control values are indicated by *$p<0.01$; **$p<0.001$. See Table 9 and page 60 for discussion of GABA significance.

	Pcs group		Hepatic devascularization group	
	Sham-operation (7)	Pcs (8)	Sham-operation	Hep. devasc.
NH_4^+	0.36 ± 0.04	0.51 ± 0.04*	0.42 ± 0.03 (21)	3.79 ± 0.44 (23)**
Glutamine	4.95 ± 0.87	12.86 ± 1.03**	5.35 ± 0.31 (18)	16.63 ± 1.04 (22)**
Glutamate	10.80 ± 0.14	9.84 ± 0.63	10.71 ± 0.25 (18)	10.14 ± 0.41 (22)
Aspartate	-	-	2.50 ± 0.07 (8)	1.72 ± 0.11 (15)**
GABA	1.97 ± 0.37	1.89 ± 0.15	2.37 ± 0.12 (12)	3.37 ± 0.28 (28)*

TABLE 7

BRAIN AMMONIA AND METABOLITES DURING GALACTOSAMINE-INDUCED ACUTE HEPATIC ENCEPHALOPATHY

Brain samples were taken 30-60 hours after the first galactosamine injection when the animals were unresponsive, or after sodium chloride injection (controls). The results are expressed in μmol/g wet weight tissue as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from control are indicated by *p<0.01; **p<0.001.

	Control	Encephalopathy
NH_4^+	0.43 ± 0.04 (6)	1.18 ± 0.15 (20)**
Glutamine	5.35 ± 0.30 (18)	13.90 ± 0.98 (21)**
Glutamate	10.39 ± 0.36 (11)	9.39 ± 0.30 (21)*
Aspartate	2.80 ± 0.26 (8)	2.50 ± 0.07 (19)
GABA	2.51 ± 0.11 (15)	2.54 ± 0.12 (21)

Significant increases in NH_4^+ and glutamine were found 65 hours after portacaval shunt alone before the onset of any obvious signs of encephalopathy, while hepatic artery ligation produced further increases in these two substances to 8-fold and 3-fold normal respectively. Very similar results were obtained in galN-induced hepatic encephalopathy (Table 7) although the increase in NH_4^+ was not as great (3-fold). These results agree with previous reports of increased brain ammonia and glutamine during acute hepatic failure in the rat (Biebuyck et al., 1974) and increased glutamine in man (Record et al., 1976). Portacaval shunt alone resulted in no other significant changes in ammonia-related metabolites. Changes in brain aspartate and glutamate concentrations during acute hepatic failure were conflicting in the two models. After hepatic devascularization there was a 31% decrease in aspartate but no significant change in glutamate (Table 6). In contrast, galN treatment resulted in slightly decreased glutamate but unchanged aspartate (Table 7). Biebuyck et al. (1974), using the devascularization method of producing acute hepatic failure, found a decrease both in aspartate and in glutamate concentrations. Both substances were decreased during acute hepatic failure in dogs (Mattson et al., 1968) while glutamate was significantly increased and aspartate unchanged in patients with fulminant hepatic failure (Record et al., 1976). From all these results and those found during chronic hepatic failure, it is apparent that although changes in either one or both of these substances is often associated with hepatic failure, these are not consistent in the various studies (see Table 8).

A significant increase was also seen in GABA in the rats with hepatic devascularization, which has not been previously reported. No

TABLE 8

SUMMARY OF CHANGES FOUND IN BRAIN ASPARTATE AND GLUTAMATE DURING HEPATIC FAILURE

Species	Condition	Aspartate	Glutamate	Reference
Rat	4 weeks pcs	↓	unchanged	Present study
Rat	3-8 weeks pcs	unchanged	↓	Hindfelt et al., 1977
Rat	8 weeks pcs + NH_4^+	↓	↓	Hindfelt et al., 1977
Rat	Hep. devasc.	↓	unchanged	Present study
Rat	GalN-necrosis	unchanged	↓	Present study
Rat	Hep. devasc.	↓	↓	Biebuyck et al., 1974
Dog	Hep. devasc.	↓	↓	Mattson et al., 1968
Man[1]	Fulminant hepatic failure	unchanged	↑	Record et al., 1976

[1]Brain samples obtained within one hour of death.

change was found in a similar study (Biebuyck et al., 1974) or in the galN model. Therefore, the possibility was considered that the change observed was the result of the delay in brain freezing since some of the rats were not sampled by freeze-blowing. Post-mortem increases in GABA have been reported when samples were not frozen within 60 seconds (Minard & Mushahwar, 1966; Alderman & Shellenberger, 1974), although others found no change even when brains were dissected on a cold stage (Nicklas & Berl, 1973). In the present studies, when values were compared from test and control animals all sampled at room temperature, a significant increase was still obtained (Table 9). When only rapidly sampled brains from test and control animals were compared the difference was only significant at the level of $p = 0.19$. No significant difference was obtained when rapidly sampled and slowly sampled brains were compared in each group. Thus, although the present results must be interpreted with caution, they do suggest that possible changes in GABA during hepatic encephalopathy cannot be ruled out.

The changes in brain concentrations of ammonia and the amino acids in the various models of acute and chronic hepatic failure compared to control rats are shown in Figure 4. It can be seen that qualitatively similar results are obtained in both chronic and acute hepatic failure. The similarity of the changes produced by the two entirely different models of acute hepatic encephalopathy is striking.

CYCLIC AMP (cAMP)

cAMP was measured only in rats sampled by freeze-blowing since rapid post-mortem increases occur (Breckenridge, 1974; Nahorski & Rogers, 1973). No significant differences in brain concentration were

TABLE 9

GABA CONCENTRATIONS IN BRAINS SAMPLED BY TWO DIFFERENT METHODS FROM RATS WITH HEPATIC DEVASCULARIZATION AND CONTROLS

Brains were sampled by freeze-blowing or at room temperature as described in the Experimental Section. GABA concentrations are given in μmol/g wet weight tissue as the mean ± S.E.M. with the number of rats in parenthesis.

	(1) freeze-blown	(2) room temp.	(3) all rats
A. Control	2.24 ± 0.09 (5)	2.48 ± 0.16 (7)	2.37 ± 0.12 (12)
B. Hepatic devascularization	2.72 ± 0.35 (6)	3.56 ± 0.28 (22)	3.37 ± 0.28 (28)
A vs. B, p:	0.19	0.042	0.009
(1) vs. (2) control group, p:	0.46		
(1) vs. (2) hep. devasc. group, p:	0.14		

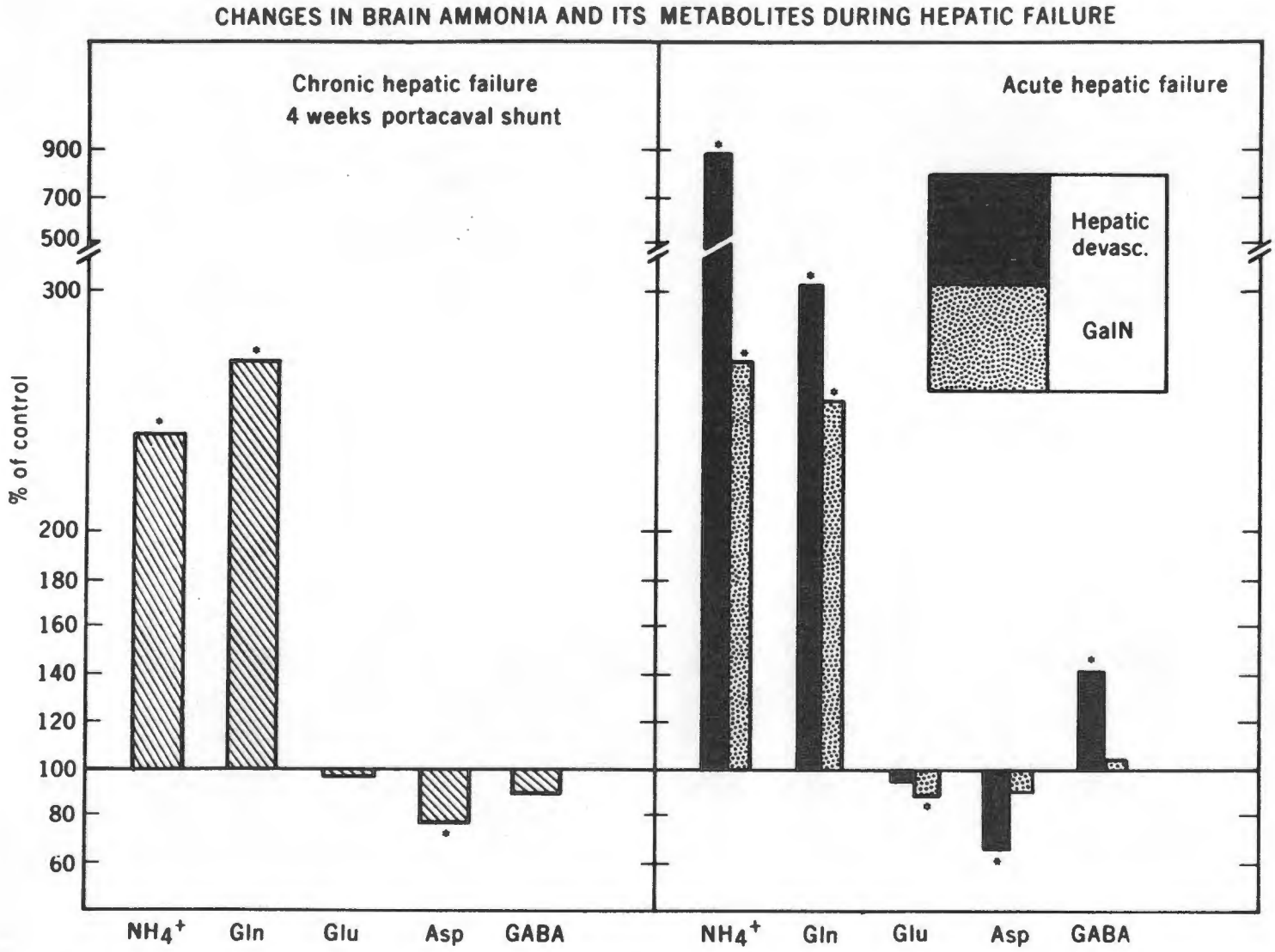
For legend to FIGURE 4, see over.

FIGURE 4

CHANGES IN BRAIN AMMONIA AND ITS METABOLITES DURING HEPATIC FAILURE

Rats were sampled four weeks after portacaval shunt (chronic hepatic failure), after hepatic devascularization or galactosamine treatment (acute hepatic failure). See the Experimental Section for further details. The results are expressed as percentage of control, as shown by the bars. Results significantly different from control values are indicated by *$p<0.01$.

FIGURE 4


CHANGES IN BRAIN AMMONIA AND ITS METABOLITES DURING HEPATIC FAILURE
Chronic hepatic failure
4 weeks portacaval shunt
Acute hepatic failure
Hepatic devasc.
GalN
% of control
900
700
500
300
200
180
160
140
120
100
80
60
NH4+
Gln
Glu
Asp
GABA
NH4+
Gln
Glu
Asp
GABA

found between control rats and those with portacaval shunt alone or hepatic devascularization (Table 10). These findings confirm those of Biebuyck et al. (1974) in a similar rat model of acute hepatic failure.

DISCUSSION

Increased brain ammonia was a prominent feature of all the forms of hepatic failure studied. The increase was greater in acute hepatic failure where brain dysfunction was more obvious than in chronic hepatic failure. However, during galN-induced encephalopathy where the rats clearly became comatose, brain ammonia concentrations were not as high as after hepatic devascularization, supporting earlier reports that the correlation with the development of encephalopathy is not exact. From other evidence it is clear that NH_4^+ per se is not the main cause of encephalopathy. However, since it is known to be toxic to the brain, and hepatic failure is closely associated with changes in brain concentrations of ammonia itself as well as its metabolites, its possible involvement in this condition cannot be ignored. Ammonia may affect the pools of its metabolites which have neuroregulatory functions, and thus influence brain activity.

The brain metabolite showing the next largest increase during hepatic failure was glutamine, which showed changes that paralleled those of NH_4^+. The size of the increase observed correlated roughly, although not absolutely, with the extent of brain dysfunction (higher concentrations were seen after hepatic artery ligation than after portacaval shunt alone, but in the galN series the coma group showed similar increases to the precoma group). Altered glutamine

TABLE 10

BRAIN CYCLIC AMP DURING ACUTE HEPATIC FAILURE

The experimental details are as described in Table 6 except that all brains were sampled by freeze-blowing. cAMP concentrations are given in nmol/g wet weight tissue as the mean ± S.E.M. with the number of rats in parenthesis. The differences between the means were not found to be significant.

Pcs group		Hepatic devascularization group	
Sham-operation (8)	Pcs (8)	Sham-operation (8)	Hep. devasc. (11)
1.55 ± 0.36	1.26 ± 0.13	1.13 ± 0.05	1.37 ± 0.29

concentrations could contribute to the development of encephalopathy in several ways. Under normal conditions glutamine may regulate the level of glutamate and consequently also of GABA (Shank & Aprison, 1977). The observation that glutaminase is an allosteric enzyme that is activated by phosphate, ammonia and nucleoside triphosphates, and inhibited by glutamate, suggests that the conversion of glutamine to glutamate is normally under fine metabolic control (Cooper, Bloom & Roth, 1978). Glutamine itself does not appear to have neurotransmitter activity. When glutamine was applied iontophoretically to nerve cells (Curtis & Crawford, 1969) or to brain slices (Bradford & McIllwain, 1966), it was found to be inactive. However, some reports suggest that glutamine may have CNS depressant effects. Cerebral glutamine levels decreased during stimulation of the mammalian CNS and parenteral glutamine tended to suppress certain features of cerebral excitation (Dobkin, 1972). Inhibition of glutamine synthesis with methionine sulphoximine can produce seizures (Sellinger & Weiler, 1963). A role for glutamine as a possible "false neurotransmitter" in hepatic coma has been suggested (Baldessarini & York, 1974). These authors found that glutamine was taken up by isolated nerve endings from rat brain and could inhibit glutamate transport. The possibility was suggested that glutamine could interfere with normal neurotransmission by accumulating in nerve endings in which glutamate is an excitatory neurotransmitter. The reports that glutamine itself is electrophysiologically inert supports this idea as do the findings that inhibition of glutamine synthetase with methionine sulphoximine can reduce the toxicity of ammonia in mice, while leading to increased levels of brain ammonia but decreased levels of glutamine (Warren &

Schenker, 1964). Even if glutamine metabolism is primarily localized in glial cells (discussed below) it is possible that excesses might be secondarily taken up by nerve terminals and accumulate there at the expense of other amino acids such as glutamate to act as a relatively inactive or "false neurotransmitter" (Baldessarini & York, 1974). Finally, glutamine may be involved in changes in blood-brain barrier function as described in the General Discussion.

Glutamate and aspartate occur in uniquely high concentrations in the brain. Their function as excitatory neurotransmitters has received much support (Johnson, 1972; Snyder et al., 1973; Curtis & Crawford, 1969; Bradford, 1970; Krnjević, 1970) but has not been clearly established. There is much evidence that glutamate is an excitatory transmitter in invertebrate systems but similar evidence for mammalian systems is deficient. Because glutamate and aspartate are also involved in intermediary metabolism it is extremely difficult to dissociate this role from the role in transmission. These amino acids are not restricted to a particular type of neuron as are acetylcholine and noradrenaline, although they are unequally distributed throughout the brain (Liebschutz et al., 1977). High affinity uptake systems have been shown to exist for glutamate and aspartate (Logan & Snyder, 1972) and when applied externally to nerve cells they exert powerful stimulatory effects on neuronal activity (Krnjević & Phillis, 1963). However, some evidence suggests that this depolarising response represents a nonspecific receptivity of the neurons to those agents and is therefore not indicative of transmitter function (Cooper et al., 1978). Others have suggested a "neuromodulator" rather than a "neurotransmitter" role for these and other neuroactive amino acids (Florey,

1967; Schrier & Thompson, 1974). Neuromodulators are described as compounds which amplify or dampen neuronal activity while neurotransmitters convey information between adjacent nerve cells (Barchas et al., 1978; Florey, 1967). Neuromodulators could be released from neurons or glia and set the "tone" of local synaptic activity by altering the effectiveness of a neurotransmitter (e.g. by affecting transmitter synthesis, release, receptor interactions, reuptake, or metabolism). Whatever the true functions of glutamate and aspartate, it is clear that changes in the concentrations of these substances may have important effects on brain function. As Table 8 shows, alterations during hepatic failure usually involve a decrease in either glutamate or aspartate or both. The close metabolic relationship between these two substances (Figure 3) may account for the finding that in one model of acute hepatic failure glutamate only was decreased while in the other it was aspartate.

It has been established that there exist at least two pools of glutamate; a small pool thought to be glial, and a larger one that is neuronal (Benjamin & Quastel, 1974; Hösli & Hösli, 1976). It is not known with any certainty how the pools relate to the various activities of this amino acid. Synthesis of GABA is thought to occur in the larger compartment which preferentially uses glucose as a substrate. Glutamine synthesis occurs in the smaller, possibly glial, compartment. Much evidence has been presented that the metabolism of glutamine is associated with glial cells (Benjamin & Quastel, 1972). It has been suggested that the glutamate is released from neurons on electrical stimulation, is taken up by the glia and converted into glutamine. Glutamine is taken up by the neurons where presumably ammonia is

released and glutamate reformed. Several findings support this suggestion:

1. ^{15}N from an administered ammonium salt is preferentially incorporated into the "small" glutamate pool that is converted into glutamine (Hindfelt, 1975).
2. The main pool of glutamine in the brain is found to be located in the glia while the major pools of glutamate, aspartate, glycine and serine are found in the neurons (Benjamin & Quastel, 1972).
3. Glutamine synthetase has been found to be localized in glial cells while none could be found in neuronal cell bodies, endothelial cells or choroid epithelium (Martinez-Hernandez, Bell & Norenberg, 1977).
4. Cultured glial cells can take up glutamate and aspartate (Hösli & Hösli, 1976; Schrier & Thompson, 1974).

Thus glial cells may play a role in modulating neuronal excitability in the CNS by regulating the extracellular level of neurotransmitters (Schrier & Thompson, 1976). In this respect it is interesting that an abundance of glial cells showing the Type II Alzheimer change is a very frequent morphological finding in chronic hepatic encephalopathy (Cavanagh, 1974; Schenker et al., 1974) in the absence of any other morphological changes. If the ammonia removing functions of glutamine synthesis were compartmented in the glia, proliferation and dilation of these cells might be a response to consistently elevated ammonia levels. Because of their situation in close proximity to or surrounding the cerebral capillaries, glial cells are the first to be exposed to ammonia as it passes into the brain.

The decarboxylated form of glutamate, GABA, is also found in large quantities in the brain (in μmole amounts compared to the nmole amounts of the monoamines). In addition, GABA is found in no other tissue but the CNS and retina. Like the monoamines it has a discrete distribution within the CNS. There is good evidence that GABA functions as an inhibitory transmitter in crustacea and a similar role has been postulated for it in mammalian systems. Considered as a whole the evidence which now favours GABA in this role is impressive although not conclusive (Curtis & Watkins, 1965; Hebb, 1970; Iversen & Kelly, 1975; Cooper et al., 1978; Krnjević, 1970). Studies on GABA uptake showed that the amino acid was selectively taken up only into certain nerve terminals, which suggested that these terminals represented those normally storing and using GABA as a transmitter (Iversen & Bloom, 1972). In addition to being a post-synaptic inhibitory transmitter, it has been postulated that GABA is involved in the production of presynaptic inhibition by depolarising nerve terminals and thereby preventing release of transmitter (Davidson, 1976). In view of these findings, the increase in concentration found after hepatic devascularization in the present study is interesting. However, as discussed in the Results section, it is not certain whether the observed increase was a true in vivo effect.

Changes in total brain content of metabolites in abnormal conditions may not accurately reflect changes in function of the various systems due to compartmentation of the metabolites into functionally separate pools. Thus, localized changes, either at the regional or cellular level, may be more significant in determining brain function. In addition, the brain undoubtedly has many homeostatic mechanisms for

the maintenance of neurotransmitter concentrations, and therefore it would seem that at least in the cases of GABA and glutamate, changes in the activities of these systems would not necessarily lead to substantial changes in total concentrations within the brain. Information about functional turnover is needed before conclusions may be drawn about their activities (Davidson, 1976; Cooper et al., 1978).

The same remarks might apply to the study of cyclic AMP which is becoming increasingly important in consideration of CNS transmission. In addition to its role as a "second messenger" (Greengard, 1976) it has recently been suggested that cAMP may function as a synaptic modulator involving glial cells (Iversen, 1974). According to this hypothesis cAMP released from the glial cells surrounding the Purkinje neurons may mediate the well-known inhibitory effects of noradrenaline on these neurons. This would involve the release of and an external site of action for cAMP which in other systems is thought of as a strictly intracellular messenger. In addition it means that the glia would be capable of responding to a neurotransmitter substance, a concept that has not been previously considered.

The uniform elevation of brain cAMP during anaesthesia caused by several diverse anaesthetic agents (Biebuyck et al., 1975) prompted the study of cAMP in another form of unconsciousness. It is of interest therefore that no significant changes in brain cAMP content were found during hepatic failure. As indicated above, however, this finding does not rule out any changes in activity of cAMP in this condition.

RESULTS SECTION II

NEUROTRANSMITTERS: RELATED TO PLASMA/BRAIN AMINO ACID RELATIONSHIPS

Alterations in brain concentrations of the neurotransmitters serotonin and the catecholamines are common findings in all forms of hepatic encephalopathy (Cummings, Soeters, James, Keane & Fischer, 1976a; Cummings, James, Soeters, Keane, Foster & Fischer, 1976b; Dodsworth et al., 1974; Curzon et al., 1975a). Concentrations of the precursors for these neurotransmitters, the essential aromatic amino acids tryptophan, tyrosine and phenylalanine, are also changed, both in brain and blood, during hepatic failure (Rosen, Soeters, James, Hodgman & Fischer, 1978; Curzon, Kantamaneni, Winch, Rojas-Bueno, Murray-Lyon & Williams, 1973b). Changes in serotonin metabolism have been especially consistent. Portacaval shunting in the rat resulted in large increases in brain tryptophan and 5-hydroxyindoleacetic acid (5-HIAA, the major product of serotonin catabolism) and smaller but significant increases in serotonin (Cummings et al., 1976a; Curzon et al., 1975a; Baldessarini & Fischer, 1973; James et al., 1976; Rosen et al., 1978). Similar changes have been found during acute hepatic failure in the rat (Fischer, James & Baldessarini, 1972; Tyce, Flock & Owen, 1967; Cummings et al., 1976b) and the pig (Curzon et al., 1973b). These findings, and the known toxicity of oral tryptophan in hepatic failure (Ogihara, Mozai & Hirai, 1966; Condon, 1971), led to the suggestion that changes in hydroxyindole metabolism in the brain are involved in the development of hepatic encephalopathy (Curzon et al., 1975a; Baldessarini & Fischer, 1973; Cummings et al., 1976a,b).

In the brain, serotonin synthesis appears to be regulated by the availability of free tryptophan, due to the low K_m of the rate-limiting enzyme in the synthetic pathway, tryptophan hydroxylase, which appears to be normally unsaturated with tryptophan (Friedman, Kappelman & Kaufman, 1972). The systemic administration of tryptophan increases whole brain concentrations of serotonin up to a maximum (Fernstrom & Wurtman, 1971a; Moir & Eccleston, 1968). Many drugs and changes in physiological variables produce parallel changes in brain tryptophan content and serotonin turnover, as estimated from changes in steady-state 5-HIAA concentrations (Tagliamonte, Tagliamonte, Pérez-Cruet, Stern & Gessa, 1971; Guerinot, Doitou & Bohuon, 1974; Pérez-Cruet, Tagliamonte, Tagliamonte & Gessa, 1971; Messing, Fisher, Phebus & Lytle, 1976; Colmenares, Wurtman & Fernstrom, 1975; Curzon, Joseph & Knott, 1972; Baldessarini & Fischer, 1973). Thus, changes in brain tryptophan concentrations may have important effects on brain serotonin levels.

Synthesis of the catecholamines, noradrenaline and dopamine, does not appear to be limited by availability of the precursor tyrosine, although it may have some influence (Wurtman, Larin, Mostafapour & Fernstrom, 1974). However, high concentrations of tryptophan and phenylalanine may inhibit catecholamine synthesis by competition (Curtius, Baerlocher & Völlmin, 1972; Andrews et al., 1978).

The aromatic amino acids are transported into the brain by a mechanism that is shared with the other large neutral amino acids leucine, isoleucine, valine, methionine, and threonine (Oldendorf, 1971b; Oldendorf & Szabo, 1976) and competition among these has been shown to affect influx of individual amino acids (Fernstrom & Faller, 1975;

Pérez-Cruet, Chase & Murphy, 1974; Etienne, Young & Sourkes, 1976). For example, a good correlation between the brain content of any one of the neutral amino acids and the ratio of its plasma concentration to the sum of the other competing neutral amino acids has been shown in individual normal rats (Fernstrom & Faller, 1978; Ashley & Anderson, 1975). Evidence of these competition effects led to suggestions that alterations in plasma amino acid patterns found during hepatic failure might be involved in causing altered brain amino acid concentrations and thus disturb brain neurotransmitter metabolism. Chronic liver disease both in man and experimental animals is characterized by a plasma pattern with high phenylalanine, methionine, tyrosine, and sometimes tryptophan, and lowered branched chain amino acids valine, leucine, and isoleucine (Fischer et al., 1974, 1975; James et al., 1976; Sherwin et al., 1974; Hirayama, 1971; Maddrey et al., 1976). It was suggested that the decreased branched chain amino acids (presumably resulting from decreased insulin degradation due to shunting of blood past the liver) could increase brain tryptophan content by decreasing competition for transport (Munro et al., 1975). High insulin concentrations would stimulate entry of the branched chain amino acids into muscle tissue where these are primarily catabolized. The aromatic amino acids on the other hand, are catabolized by the liver and would accumulate during hepatic failure. Some evidence for increased plasma insulin during chronic hepatic failure has been presented (Creutzfeldt, Frerichs & Sickinger, 1970; Marco, Diego, Villanueva, Diaz-Fierros, Valverde & Segovia, 1973). Others have suggested that it is the low insulin to glucagon ratio which promotes the abnormal plasma amino acid pattern (Soeters & Fischer, 1976). High glucagon levels have

been found during chronic hepatic failure in man (Sherwin et al., 1974) and after portacaval shunt in the dog (Soeters, Weir, Ebeid & Fischer, 1976a). The hypothesis was supported by the finding that administration of branched chain amino acids to dogs with portacaval shunts led to improved mental status as well as normalized plasma amino acids (Fischer, Funovics, Aguirre, James, Keane, Wesdorp, Yoshimura & Westman, 1975). One objection, however, is that in most studies the decreases in the branched chain amino acids were very small in comparison to the increases in phenylalanine and tyrosine which are also competitors of tryptophan transport. Indeed it has been reported that phenylalanine and tyrosine are stronger competitors of tryptophan transport in vivo than the branched chain amino acids (Daniel, Love, Moorhouse & Pratt, 1975b).

An entirely different situation exists during acute hepatic failure. Recent studies have revealed qualitative and quantitative differences in the plasma amino acids in animals with encephalopathy from acute and chronic liver disease (Record et al., 1976; Rosen, Yoshimura, Hodgman & Fischer, 1977). Acute hepatic failure led to large elevations in most of the plasma amino acids studied (McMenamy, Vang & Drapanas, 1965; Record et al., 1976). In contrast to the consistent reports of decreased branched chain amino acids in chronic hepatic failure, the findings in acute hepatic failure have been conflicting. In fulminant hepatic failure in man the branched chain amino acids were found to be decreased (Knell, Pratt, Curzon & Williams, 1972) or normal (Record et al., 1976) while in experimental acute hepatic failure in the pig they were unchanged (Buxton et al., 1974).

Another factor which appears to influence tryptophan uptake in many circumstances is the concentration of unbound tryptophan in the plasma (Tagliamonte, Biggio, Vargui & Gessa, 1973b; Curzon, Knott, Murray-Lyon, Record & Williams, 1975b). Tryptophan is unique among free plasma amino acids in that it is reversibly bound to albumin, normally 80-90% (McMenamy & Oncley, 1958). Many physiological factors affect the binding constant, several of them mediated by changes in the plasma free fatty acids which release tryptophan from its binding sites (Curzon & Knott, 1974b; Curzon, Friedel, Kantamaneni, Greenwood & Lader, 1974; Lipsett, Madras, Wurtman & Munro, 1973). Unbound tryptophan levels are often increased in hepatic failure and changes in brain tryptophan have been correlated with unbound tryptophan levels in man (Knell et al., 1974) and animals (Curzon et al., 1973b; Buxton et al., 1974) with acute hepatic failure. However, its relationship with brain does not appear to be simple (Madras et al., 1974) and it is most likely that several factors are acting together to regulate tryptophan transport into the brain.

In this section of the thesis, studies of plasma and brain amino acid concentrations and their relationships (with particular respect to brain tryptophan content) during hepatic failure, are described.

INFLUENCE OF DIET

Plasma and brain tryptophan and brain serotonin concentrations have been shown to vary diurnally (Héry, Rouer & Glowinski, 1972; Wurtman & Fernstrom, 1972) primarily due to food intake (Wurtman & Fernstrom, 1974). Therefore, attempts were made to sample the rats in each group, including the controls, at the same time of day.

The chronic hepatic failure rats were pair-fed with controls to eliminate food intake differences, and were sampled between 8 and 10 a.m.

In the acute hepatic failure studies, the rats were sampled 65 hours after portacaval shunt, between 8 and 10 a.m., or during the afternoon 4-7 hours after ligation of the hepatic artery. The latter group had no access to food after hepatic artery ligation, their blood glucose concentrations being maintained by dextrose infusion. In addition, one experimental group of rats was deprived of all food after portacaval shunt, and allowed access to water only, until after hepatic artery ligation when blood glucose maintenance was begun. The aim of this experiment was to determine if dietary differences between the shunted rats and controls (due to decreased food intake by the shunted rats) could be responsible for the changes observed in brain and plasma metabolites. The results showed that changes in brain and plasma metabolites did not depend on whether rats were fed or starved between shunting and ligation.

RESULTS

1. BRAIN AMINO ACIDS

Four weeks after portacaval shunt, concentrations of the aromatic amino acids tryptophan, tyrosine and phenylalanine were significantly increased in the brain by 60 to 107% (Table 11). Similar changes have been previously reported in the portacaval shunted rat (Curzon et al., 1975a; James et al., 1976; Cummings et al., 1976a). The other neutral amino acids measured were unchanged during chronic hepatic failure.

In the hepatic devascularization series (Table 12, Figure 5) only tryptophan was increased 65 hours after portacaval shunt alone, while ligation of the hepatic artery produced a further increase in tryptophan to nearly 2-fold and also large increases in tyrosine (5-fold) and phenylalanine (3-fold). In addition, brain alanine content was doubled.

Galactosamine-induced acute hepatic encephalopathy led to very similar brain changes (Table 13). Tryptophan was nearly doubled, tyrosine increased 6-fold and phenylalanine doubled in the coma group. In addition, concentrations of methionine, isoleucine, leucine and glycine showed significant increases, which were not seen in the surgical model. In Figure 6 the changes in the precoma and coma groups are compared to the control values. All significant changes seen were more marked during coma than precoma (with the exception of leucine).

Thus, both forms of acute hepatic encephalopathy were characterised by brain increases in the essential aromatic amino acids which became more pronounced as brain dysfunction increased.

TABLE 11

BRAIN AMINO ACIDS DURING CHRONIC HEPATIC FAILURE

All rats were sampled four weeks after portacaval shunt (pcs) or sham-operation (control). The results are expressed in µmol/g wet weight tissue as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from control are indicated by *p<0.05, **p<0.001.

	Control	Pcs
Tryptophan	0.024 ± 0.002 (11)	0.051 ± 0.003 (13)**
Tyrosine	0.084 ± 0.009 (11)	0.17 ± 0.02 (8)**
Phenylalanine	0.097 ± 0.020 (11)	0.16 ± 0.02 (8)*
Methionine	0.054 ± 0.012 (7)	0.053 ± 0.005 (7)
Valine	0.092 ± 0.011 (10)	0.098 ± 0.010 (6)
Leucine	0.11 ± 0.01 (11)	0.12 ± 0.01 (9)
Isoleucine	0.060 ± 0.010 (11)	0.047 ± 0.002 (9)

TABLE 12

BRAIN AMINO ACIDS AFTER PORTACAVAL SHUNT ALONE AND AFTER TWO-STAGE HEPATIC DEVASCULARIZATION

Samples were taken from two groups of rats; following portacaval shunt (pcs), and following portacaval shunt and hepatic artery ligation (hepatic devascularization). Each group was compared to the corresponding controls (see Experimental Section for further details). The results are expressed in μmol/g wet weight tissue as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from control values are indicated by *p<0.05; **p<0.001.

	Pcs group		Hepatic devascularization group	
	Sham-operation	Pcs	Sham-operation	Hep. devasc.
Tryptophan	0.030 ± 0.003 (7)	0.044 ± 0.003 (6)*	0.029 ± 0.001 (20)	0.054 ± 0.002 (23)**
Tyrosine	0.079 ± 0.012 (7)	0.12 ± 0.03 (7)	0.071 ± 0.010 (13)	0.33 ± 0.03 (20)**
Phenylalanine	0.058 ± 0.015 (7)	0.11 ± 0.03 (8)	0.088 ± 0.010 (12)	0.29 ± 0.03 (19)**
Methionine	0.017 (1)	0.054 (2)	0.058 ± 0.030 (9)	0.11 ± 0.01 (17)
Valine	0.037 ± 0.011 (4)	0.089 ± 0.025 (6)	0.089 ± 0.020 (10)	0.084 ± 0.010 (15)
Leucine	0.062 ± 0.006 (6)	0.059 ± 0.011 (8)	0.086 ± 0.010 (13)	0.11 ± 0.01 (21)
Isoleucine	0.030 ± 0.011 (7)	0.041 ± 0.006 (7)	0.054 ± 0.010 (14)	0.058 ± 0.013 (20)
Threonine	-	-	1.4 ± 0.3 (6)	1.7 ± 0.7 (11)
Glycine	0.58 ± 0.06 (8)	0.60 ± 0.06 (8)	0.69 ± 0.08 (14)	0.77 ± 0.07 (20)
Alanine	0.39 ± 0.06 (8)	0.31 ± 0.04 (8)	0.36 ± 0.05 (14)	0.80 ± 0.11 (21)*

For legend to FIGURE 5, see over.

FIGURE 5

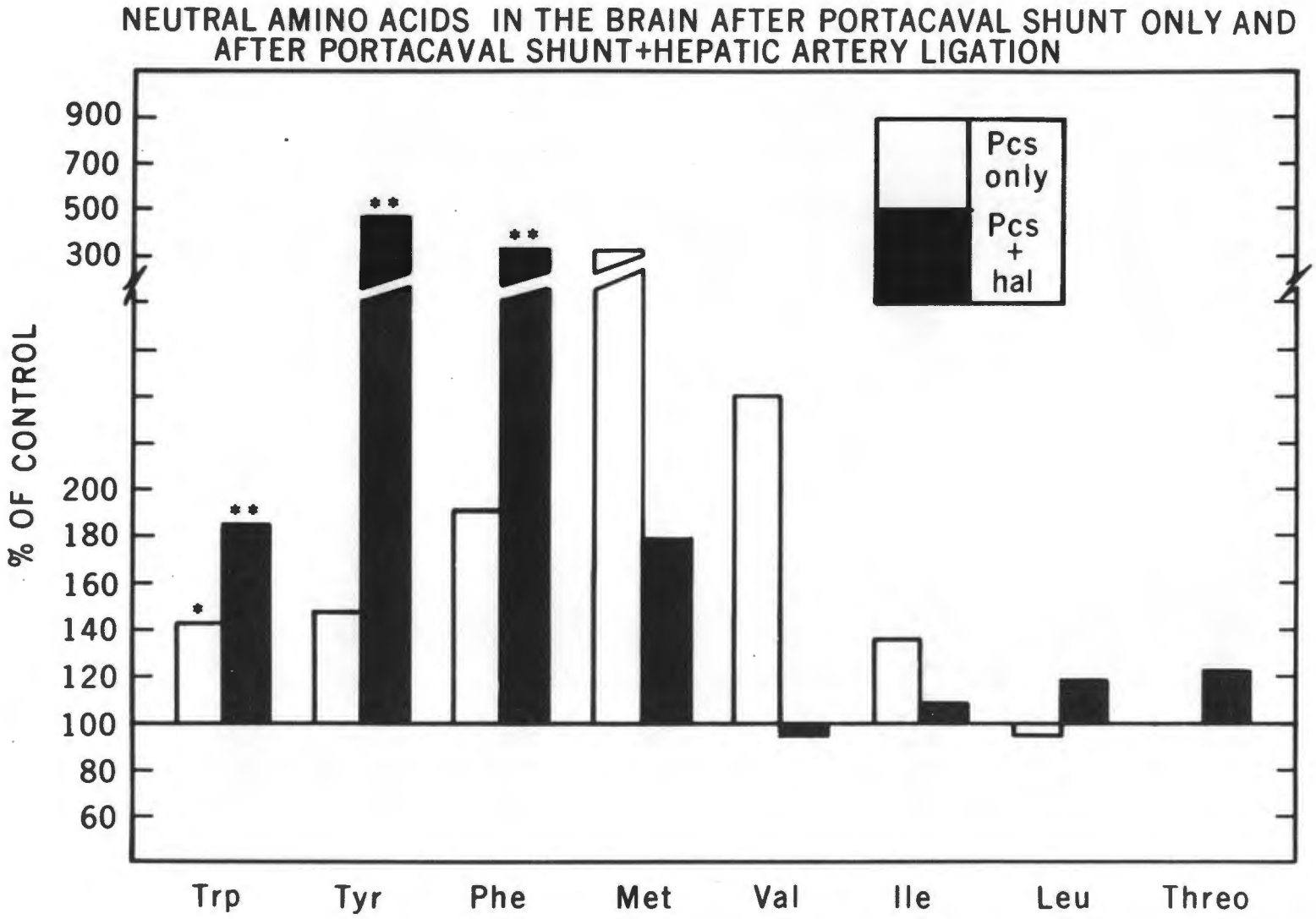

NEUTRAL AMINO ACIDS IN THE BRAIN AFTER PORTACAVAL SHUNT ONLY AND AFTER PORTACAVAL SHUNT+HEPATIC ARTERY LIGATION
% OF CONTROL
900
700
500
300
200
180
160
140
120
100
80
60
Pcs only
Pcs + hal
Trp
Tyr
Phe
Met
Val
Ile
Leu
Threo

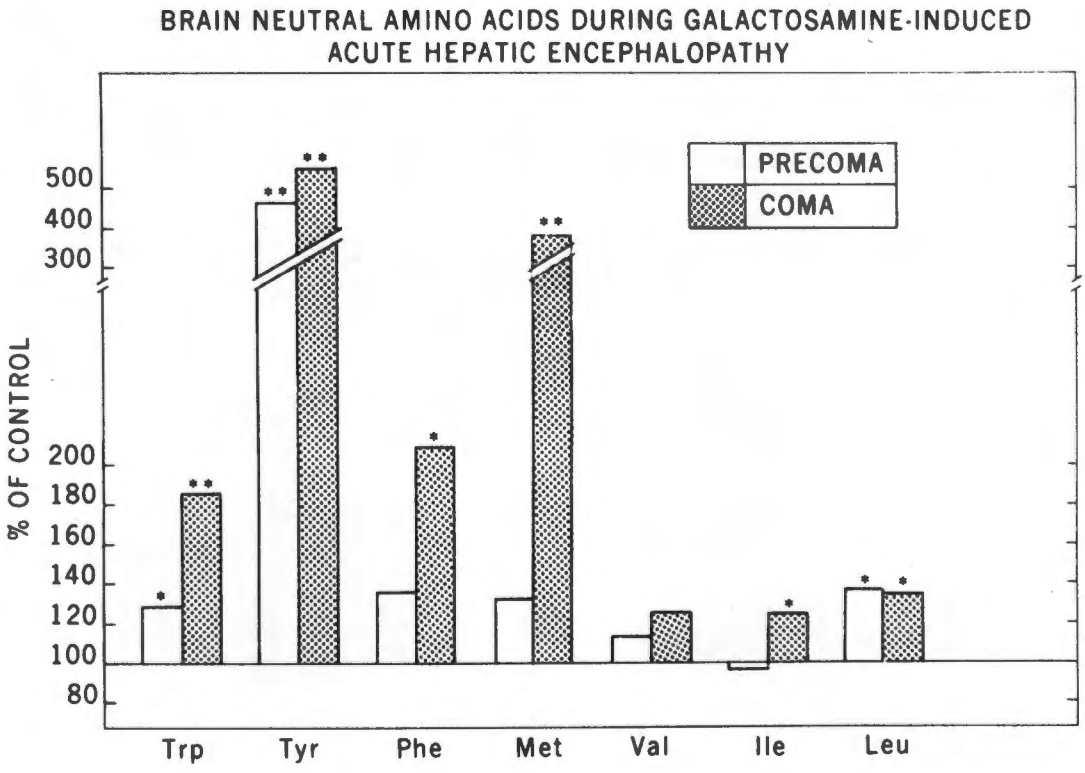
TABLE 13

EFFECT OF GALACTOSAMINE-INDUCED HEPATIC ENCEPHALOPATHY ON BRAIN AMINO ACIDS

Samples were taken 30-60 hours after the first galactosamine injection when the animals were either in a precomatose or comatose state (see Experimental Section for definitions). The results are expressed as the mean ± S.E.M. in μmol/g wet weight tissue with the number of rats in parenthesis. Results significantly different from control values are indicated by *$p<0.05$, **$p<0.01$; significant differences between the precoma and coma groups by $^{+}p<0.05$.

	Control	Precoma	Coma
Tryptophan	0.033 ± 0.001 (8)	0.043 ± 0.006 (8)*	0.061 ± 0.004 (8)**+
Tyrosine	0.086 ± 0.010 (7)	0.40 ± 0.06 (8)**	0.56 ± 0.05 (12)**+
Phenylalanine	0.11 ± 0.01 (6)	0.15 ± 0.03 (8)	0.22 ± 0.04 (11)*
Methionine	0.037 ± 0.010 (6)	0.049 ± 0.010 (5)	0.14 ± 0.02 (11)**+
Valine	0.16 ± 0.03 (6)	0.17 ± 0.02 (8)	0.19 ± 0.01 (11)
Leucine	0.11 ± 0.01 (7)	0.15 ± 0.01 (8)*	0.15 ± 0.01 (13)*
Isoleucine	0.063 ± 0.010 (7)	0.060 ± 0.010 (8)	0.080 ± 0.010 (13)*+
Glycine	0.84 ± 0.11 (7)	1.2 ± 0.2 (8)*	1.4 ± 0.1 (13)*
Alanine	0.52 ± 0.07 (7)	0.59 ± 0.07 (8)	0.65 ± 0.07 (13)

FIGURE 6



The rats were sampled 30-60 hours after the first galactosamine injection when the rats were either in a precomatose or a comatose state, as described in the Experimental Section. The bars represent percentage of control, with the results significantly different from control values indicated by *$p<0.05$ and **$p<0.01$.

Increased brain tryptophan has been observed during experimental acute hepatic failure in animals (Curzon et al., 1973b; Mattson et al., 1968; Tyce et al., 1967; Cummings et al., 1976b) and fulminant hepatic failure in man (Record et al., 1976). Increases in other nonessential and essential amino acids, including the branched chain amino acids, were reported in the dog after hepatic devascularization (Mattson et al., 1968) and in patients with fulminant hepatic failure (Record et al., 1976).

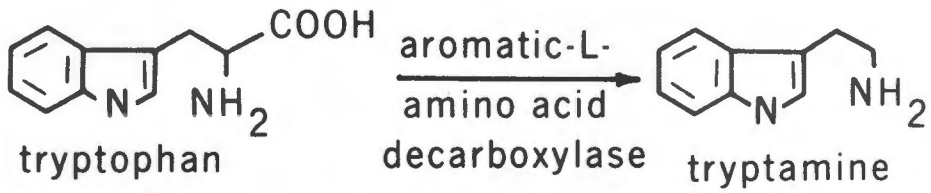
DISCUSSION

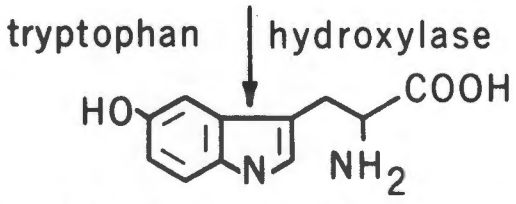
In all three conditions studied, particularly striking increases were seen in brain concentrations of the aromatic amino acids tryptophan and tyrosine, which are neurotransmitter precursors, and phenylalanine, which may also influence neurotransmitter synthesis in the brain (Curtius et al., 1972; Bagchi & Zarycki, 1973). Acute hepatic failure resulted in much larger increases than chronic hepatic failure. The three branched-chain amino acids on the other hand, which share the blood-brain barrier transport system with the aromatic amino acids, were unaltered except during galactosamine coma when isoleucine and leucine were raised.

The increase in brain tryptophan content is of special significance because there is considerable evidence that brain tryptophan availability is a major factor regulating the rate of serotonin synthesis in the central nervous system (see Figure 7). In vitro determination of the K_m of the first enzyme of the pathway, tryptophan hydroxylase, suggested that the enzyme is unsaturated with substrate at normal cerebral tryptophan concentrations in the presence of the

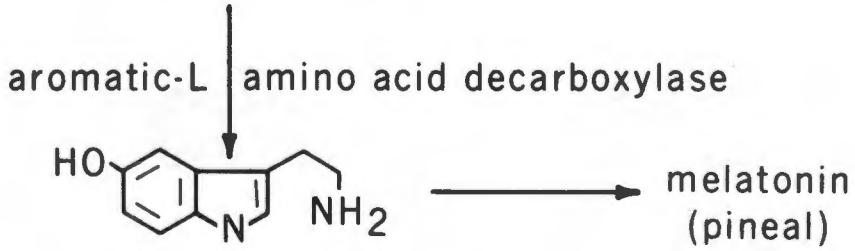
FIGURE 7

METABOLISM OF SEROTONIN IN THE BRAIN

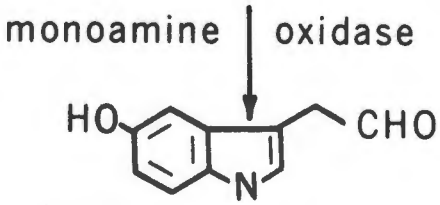




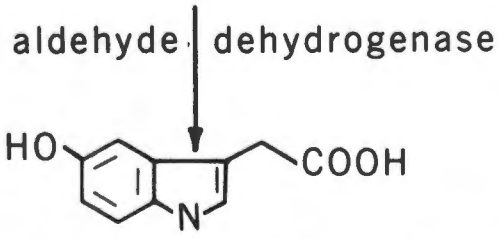
5-hydroxy tryptophan



5-hydroxytryptamine (serotonin)



5-hydroxyindolealdehyde



5-hydroxyindoleacetic acid (5-HIAA)

natural cofactor, tetrahydrobiopterin (Friedman et al., 1972). The K_m for tryptophan was found to be about 50 µM which is in the range of normal brain tryptophan concentrations (Costa & Meek, 1974). This implies that an increase in the concentration of tryptophan available to the enzyme would be reflected in an increase in the synthesis of 5-hydroxytryptophan. Since there is no evidence that the decarboxylation of 5-hydroxytryptophan to serotonin is rate-limiting, and there is normally a considerably greater activity of aromatic-L-amino acid decarboxylase than of tryptophan hydroxylase in brain tissue (Ichiyama, Nakamura, Nishizuka & Hayaishi, 1968) this would lead to an increase in the synthesis of serotonin. In vivo studies support this view; an increase in cerebral tryptophan concentration has been shown to lead to an increase in the rate of serotonin synthesis as indicated by increased serotonin and 5-HIAA concentrations (Carlsson, 1974; Gessa & Tagliamonte, 1974; Curzon et al., 1975a; Bender, 1976). Some evidence suggests that factors other than tryptophan availability affect serotonin synthesis in the brain (Bender, 1976; Mandell, 1978) and feed-back inhibition has been suggested (Macon, Sokoloff & Glowinski, 1971; Hamon, Bourgoin & Glowinski, 1973) but this has not been demonstrated in vivo and the validity of the experiments have been questioned on technical grounds (Green & Graham-Smith, 1976). It is somewhat surprising that the synthesis of a substance of importance for brain function should be influenced by the many factors which can alter brain tryptophan concentration and indeed Graham-Smith (1974) has suggested that these factors may normally have little influence on the amount of brain serotonin at receptor sites. Evidence that brain serotonin exists in two or more pools (Shields & Eccleston, 1973; Lane & Aprison,

1978) implies the possibility that total brain serotonin and functional brain serotonin are not related. In addition, tryptophan availability for serotonin synthesis may depend not only on total brain tryptophan concentrations but also on the neuronal membrane transport system which may be limiting for serotonin synthesis in some circumstances (Mandell, 1978).

Some indirect evidence for the importance of tryptophan availability to serotonin synthesis and function has been presented. Changes in sensitivity to pain, which has been linked to changes in brain serotonin concentrations, have also been correlated with changes in brain tryptophan (Tenin, 1967; Lytle, Messing, Fisher & Phebus, 1975). Administration of tryptophan has also been shown to modify sleep (Hartmann, Chung & Chien, 1971) as well as the secretion of prolactin and gonadotropins in humans (MacIndoe & Turkington, 1973). Both these body functions have been shown to be influenced by serotoninergic neurons.

The role of serotoninergic mechanisms in central nervous system function is still a matter of much discussion, and the exact relationship of central indoleamines to the diverse cerebral activities and pathophysiology of several diseases with which they have been linked is not clear (see Chase & Murphy, 1973; Holman, Elliott & Barchas, 1975). Considerable biochemical evidence supports a special relationship between serotoninergic mechanisms and the maintenance of non-REM sleep (Jouvet, 1969, 1972; Pujol, Buguet, Froment, Jones & Jouvet, 1978; Holman et al., 1975). In a recent review Gillin, Mendelson, Sitaram & Wyatt (1978) reported that much of the evidence suggests that serotonin is involved in regulation of states of consciousness. Work by Jouvet (1974) has shown that serotonin is inhibitory and noradrenaline

excitatory in the central arousal system (reticular activating system) of cats. Therefore the finding of increased brain serotonin and 5-HIAA during acute and chronic hepatic encephalopathy (Cummings et al., 1976a,b; Curzon et al., 1973b; Tyce et al., 1967) stimulated interest in 5-hydroxyindole metabolism in hepatic failure. The importance of the changes found in 5-HIAA concentrations was recently emphasized when it was reported that serotonin is only metabolized to 5-HIAA after release into the synapse and reuptake, strongly suggesting that changes in 5-HIAA concentrations do imply changes in the functional concentrations of serotonin (Reinhard & Wurtman, 1977). Further evidence for the importance of tryptophan metabolism in hepatic encephalopathy was obtained from experiments with portacaval shunted rats given tryptophan (Bloxam, Curzon, Kantamaneni & Tricklebank, 1977). These rats showed impaired ambulatory behaviour compared to controls.

In the present study, brain tryptophan was increased 47% 65 hours after portacaval shunt before ligation of the hepatic artery and any obvious signs of encephalopathy. After the onset of encephalopathy 4-7 hours after hepatic artery ligation, the level had increased 86% as compared to controls. Similarly, in the galactosamine model tryptophan was increased 30% in the precoma group and 85% in the coma group. Thus increasing brain tryptophan was a characteristic feature during the development of encephalopathy. The increased concentrations observed after shunting alone, where brain dysfunction was not apparent, may indicate that tryptophan is associated with the development of encephalopathy only in combination with other changes, or after a critical level in the brain has been reached. Alternatively, brain damage may be present in this condition but not so as to cause

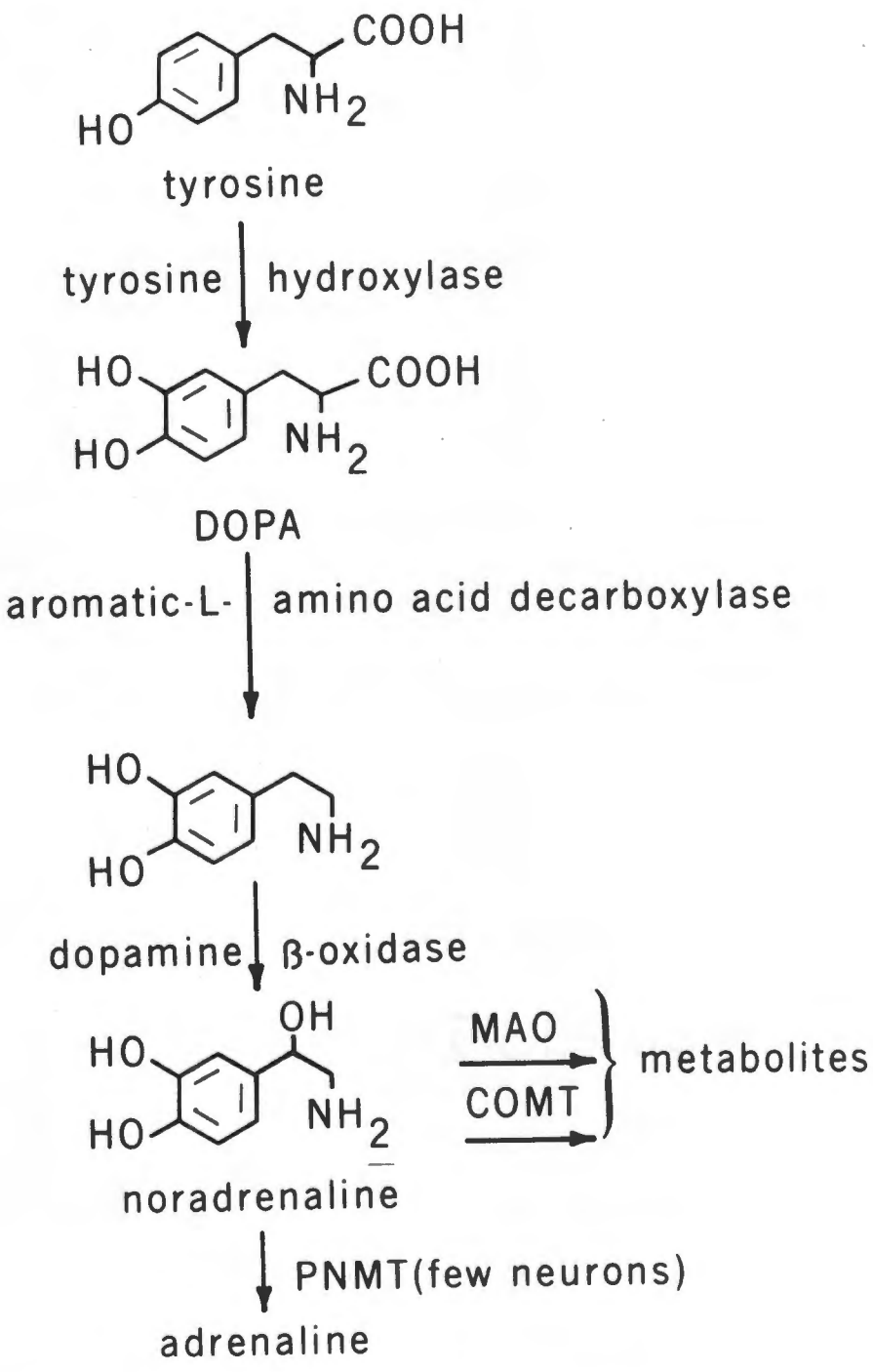
noticeable changes in behaviour of the rat. The early stages of coma as defined in patients involve impairment of several brain functions that must be specifically tested for and which would not be obvious in the rat.

Metabolites of tryptophan may also influence a completely different system in the brain, namely that of glucose metabolism. Treatment of rats with either 5-hydroxytryptophan or an inhibitor of monoamine oxidase (both leading to increased brain serotonin) or both, seemed to impair the transport of glucose from plasma to brain, and significantly reduced the utilization of glucose in brain tissue (Wong & Tyce, 1978). Possible relationships between aromatic amino acids and other metabolic pathways are discussed further in the General Discussion.

Brain concentrations of noradrenaline, which has mostly excitatory effects in the central nervous system (Baldessarini, 1972), are decreased in acute hepatic coma (Dodsworth et al., 1974). As with serotonin, the changes found in brain aromatic amino acids may be related to this finding. The influence of brain tryptophan on serotonin biosynthesis is in marked contrast to the relationship between tyrosine and brain catecholamine synthesis, which appears to be regulated mainly by endproduct inhibition (Costa & Meek, 1974; Baldessarini & Karobath, 1973). Since the rate-limiting enzyme, tyrosine hydroxylase (see Figure 8) is almost completely saturated when the concentration of the substrate is in the normal range (McGeer, McGeer & Wada, 1971), changes in brain concentrations of tyrosine would not be expected to affect catecholamine synthesis. However, some evidence has been presented that tyrosine can and may control the rates of catecholamine

FIGURE 8

BRAIN CATECHOLAMINE SYNTHESIS



MAO=monoamine oxidase
COMT=catechol-O-methyl transferase
PNMT = phenylethanolamine-N-methyl transferase

synthesis (Wurtman & Fernstrom, 1975). Thus, the effects of increased brain tyrosine cannot be predicted at this stage.

The high phenylalanine concentrations found during hepatic encephalopathy may compete with tyrosine and inhibit tyrosine hydroxylation (Curtius et al., 1972); however, the increases observed in brain tyrosine (which were even greater than phenylalanine, Figures 5 and 6) would be expected to overcome this effect. On the other hand, the nonspecificity of the next enzyme in the biosynthetic pathway, aromatic-L-amino acid decarboxylase, may lead to inhibition of catecholamine synthesis at this step by the high concentrations of phenylalanine, tryptophan, 5-hydroxytryptophan and serotonin (Andrews et al., 1978; Cooper et al., 1978). Such inhibition may result in the decreased brain noradrenaline concentrations found during acute hepatic failure (Dodsworth et al., 1974).

2. PLASMA AMINO ACIDS

Plasma unbound and total tryptophan concentrations were unchanged four weeks after portacaval shunt (Table 14). Of the other aromatic amino acids, only tyrosine was increased (190% of control value). Two of the branched chain amino acids, leucine and isoleucine, were significantly decreased to 73% of control values. These changes have been previously seen three to four weeks after portacaval shunt in the rat; however, earlier reports of increased unbound tryptophan or phenylalanine were not confirmed (Curzon et al., 1975a; James et al., 1976; Cummings et al., 1976a).

In marked contrast to the chronic condition were the plasma changes during acute hepatic failure. In the surgical model (Table 15, Figure 9) plasma concentrations of tyrosine and phenylalanine had doubled 65 hours after portacaval shunt alone. After ligation of the hepatic artery there were striking increases in all the amino acids measured, with the exception of total tryptophan which was decreased to 81% of control. Unbound tryptophan had doubled while the other aromatic amino acids tyrosine and phenylalanine were increased 7-fold and 6-fold respectively. Methionine showed a similar increase. The branched chain amino acid concentrations had doubled. The non-essential amino acids serine, glycine and threonine were increased three- to five-fold and alanine showed a 10-fold increase.

Changes during galN-induced hepatic encephalopathy (Table 16) were very similar to those seen after hepatic devascularization with even greater decreases (in the case of total tryptophan) and increases (in the case of all the other amino acids). No significant differences were found between the plasma amino acids in the precoma and coma

TABLE 14

PLASMA AMINO ACIDS DURING CHRONIC HEPATIC FAILURE

All rats were sampled four weeks after portacaval shunt (pcs) or sham-operation (control). The results are expressed in μmol/ml plasma as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from control are indicated by *p<0.02, **p<0.01.

	Control (12)	Pcs (12)
Tryptophan - unbound	0.012 ± 0.002	0.011 ± 0.003
Tryptophan - total	0.20 ± 0.01	0.18 ± 0.02
Tyrosine	0.051 ± 0.010	0.097 ± 0.010**
Phenylalanine	0.073 ± 0.010	0.091 ± 0.010
Methionine	0.047 ± 0.004	0.056 ± 0.011
Valine	0.17 ± 0.01	0.16 ± 0.02
Leucine	0.15 ± 0.01	0.11 ± 0.01*
Isoleucine	0.069 ± 0.005	0.051 ± 0.005*
Glycine	0.34 ± 0.03	0.32 ± 0.03
Alanine	0.31 ± 0.03	0.40 ± 0.08

TABLE 15

PLASMA AMINO ACIDS AFTER PORTACAVAL SHUNT ALONE AND AFTER HEPATIC DEVASCULARIZATION

Refer to Table 12 for experimental details. The results are expressed in μmol/ml as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from control are indicated by *p<0.05, **p<0.001.

	Pcs group		Hepatic devascularization group	
	Sham-operation	Pcs	Sham-operation	Hep. devasc.
Tryptophan-unbound	0.012 ± 0.002 (8)	0.0086 ± 0.001 (6)	0.017 ± 0.002 (19)	0.034 ± 0.002 (20)**
Tryptophan-total	0.18 ± 0.02 (8)	0.17 ± 0.02 (7)	0.21 ± 0.01 (19)	0.17 ± 0.01 (20)**
Tyrosine	0.063 ± 0.011 (8)	0.13 ± 0.01 (9)*	0.059 ± 0.010 (14)	0.41 ± 0.06 (15)**
Phenylalanine	0.060 ± 0.011 (8)	0.13 ± 0.01 (9)*	0.070 ± 0.010 (14)	0.42 ± 0.06 (16)**
Methionine	0.050 ± 0.008 (9)	0.067 ± 0.007 (9)	0.043 ± 0.010 (15)	0.28 ± 0.03 (18)**
Valine	0.21 ± 0.05 (8)	0.15 ± 0.02 (9)	0.17 ± 0.02 (15)	0.31 ± 0.02 (17)**
Leucine	0.14 ± 0.02 (8)	0.14 ± 0.01 (9)	0.13 ± 0.01 (15)	0.25 ± 0.01 (18)**
Isoleucine	0.067 ± 0.010 (8)	0.064 ± 0.006 (9)	0.061 ± 0.009 (15)	0.15 ± 0.02 (18)**
Threonine	0.17 ± 0.02 (4)	0.18 ± 0.03 (4)	0.15 ± 0.01 (9)	0.55 ± 0.01 (10)**
Glycine	0.45 ± 0.13 (9)	0.41 ± 0.04 (9)	0.26 ± 0.03 (15)	1.4 ± 0.1 (17)**
Alanine	0.48 ± 0.09 (9)	0.49 ± 0.09 (9)	0.26 ± 0.02 (15)	2.8 ± 0.3 (17)**
Serine	0.18 ± 0.03 (4)	0.20 ± 0.02 (4)	0.18 ± 0.01 (7)	0.75 ± 0.1 (9)**

For legend to FIGURE 9, see over.

FIGURE 9

PLASMA NEUTRAL AMINO ACIDS AFTER PORTACAVAL SHUNT ONLY AND AFTER HEPATIC DEVASCULARIZATION

Plasma samples were obtained after portacaval shunt only (pcs only) or after hepatic devascularization (pcs + hal). The results were compared to sham-operated controls. For further details see the Experimental Section. The results are shown as percentage of control values, as indicated by the bars. Results significantly different from control are indicated by *$p<0.05$ and **$p<0.001$.

FIGURE 9

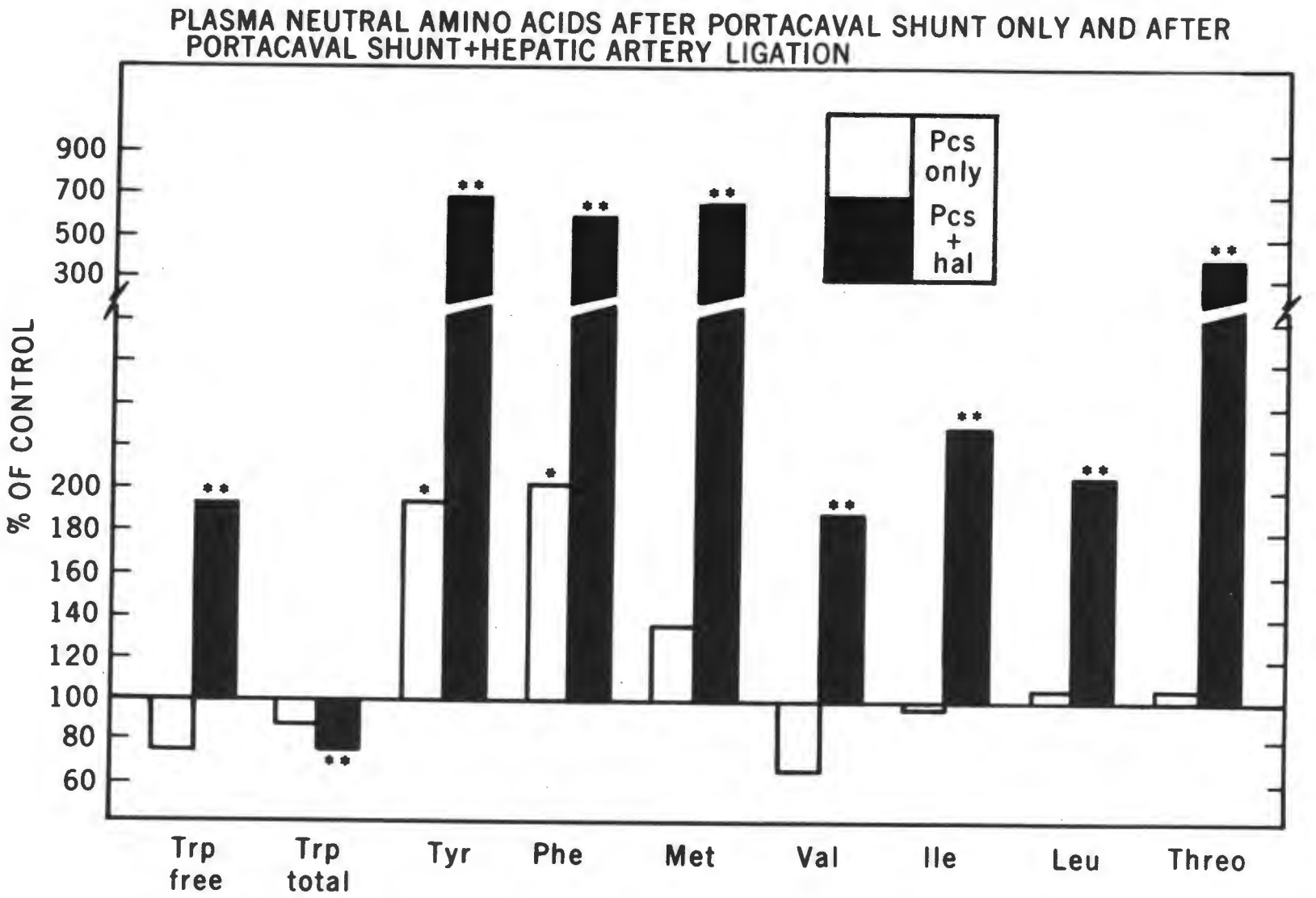

PLASMA NEUTRAL AMINO ACIDS AFTER PORTACAVAL SHUNT ONLY AND AFTER PORTACAVAL SHUNT+HEPATIC ARTERY LIGATION
% OF CONTROL
900
700
500
300
200
180
160
140
120
100
80
60
Pcs only
Pcs + hal
Trp free
Trp total
Tyr
Phe
Met
Val
Ile
Leu
Threo

TABLE 16

PLASMA AMINO ACIDS DURING GALACTOSAMINE-INDUCED HEPATIC ENCEPHALOPATHY

The experimental details are as given in Table 13. The precoma and coma groups were grouped together for this analysis. The results are expressed as mean ± S.E.M. in μmol/ml with the number of rats in parenthesis. Results significantly different from control are indicated by *p<0.01, **p<0.001.

	Control	Encephalopathy
Tryptophan-unbound	0.018 ± 0.002 (12)	0.038 ± 0.006 (12)**
Tryptophan-total	0.20 ± 0.01 (12)	0.14 ± 0.01 (13)**
Tyrosine	0.063 ± 0.010 (8)	0.86 ± 0.13 (15)**
Phenylalanine	0.080 ± 0.011 (8)	0.35 ± 0.06 (15)**
Methionine	0.043 ± 0.005 (9)	0.39 ± 0.05 (15)**
Valine	0.16 ± 0.01 (9)	0.55 ± 0.05 (15)**
Leucine	0.15 ± 0.01 (9)	0.47 ± 0.06 (15)**
Isoleucine	0.068 ± 0.010 (9)	0.24 ± 0.03 (15)**
Glycine	0.29 ± 0.03 (9)	1.1 ± 0.2 (15)*
Alanine	0.24 ± 0.02 (9)	1.8 ± 0.4 (14)**

groups; therefore these results were grouped together. Figure 10 shows the plasma neutral amino acids after hepatic devascularization and galN-induced encephalopathy as compared to control values. The similarity in the plasma changes between the two models is striking and again supports the assumption that these changes are due to a failing liver, and not the method used to produce it.

Large increases in unbound tryptophan and the aromatic and straight-chain amino acids have been previously observed during acute hepatic failure in man (Knell et al., 1974; Record et al., 1974, 1976) and experimental animals (McMenamy et al., 1965; Iob, Mattson, Sloan, Coon, Turcotte & Child, 1970; Buxton et al., 1974). Reports of total tryptophan changes have been inconsistent. Some authors found increases (Fischer et al., 1976), and others found no change (Cummings et al., 1976b; Buxton et al., 1974; Knell et al., 1974; Record et al., 1976), or decreases (Curzon et al., 1973b).

PLASMA INSULIN AND GLUCAGON DURING ACUTE HEPATIC FAILURE

Plasma insulin concentrations in both models of acute hepatic failure were not significantly different from those in control rats (Table 17). However, glucagon concentrations had increased 6-fold after hepatic devascularization and 4-fold in the rats treated with galN, resulting in a greatly decreased insulin to glucagon ratio.

Slightly elevated circulating insulin levels have been found in association with cirrhosis (Creutzfeld et al., 1970; Marco et al., 1973). In agreement with the above findings a much larger increase in plasma glucagon than insulin was found after portacaval shunt

For legend to FIGURE 10, see over.

FIGURE 10

PLASMA NEUTRAL AMINO ACIDS DURING ACUTE HEPATIC FAILURE

The experimental details are as given for Figures 6 and 9. The bars indicate percentage of control values with significantly different results indicated by *$p<0.01$ and **$p<0.001$.

FIGURE 10

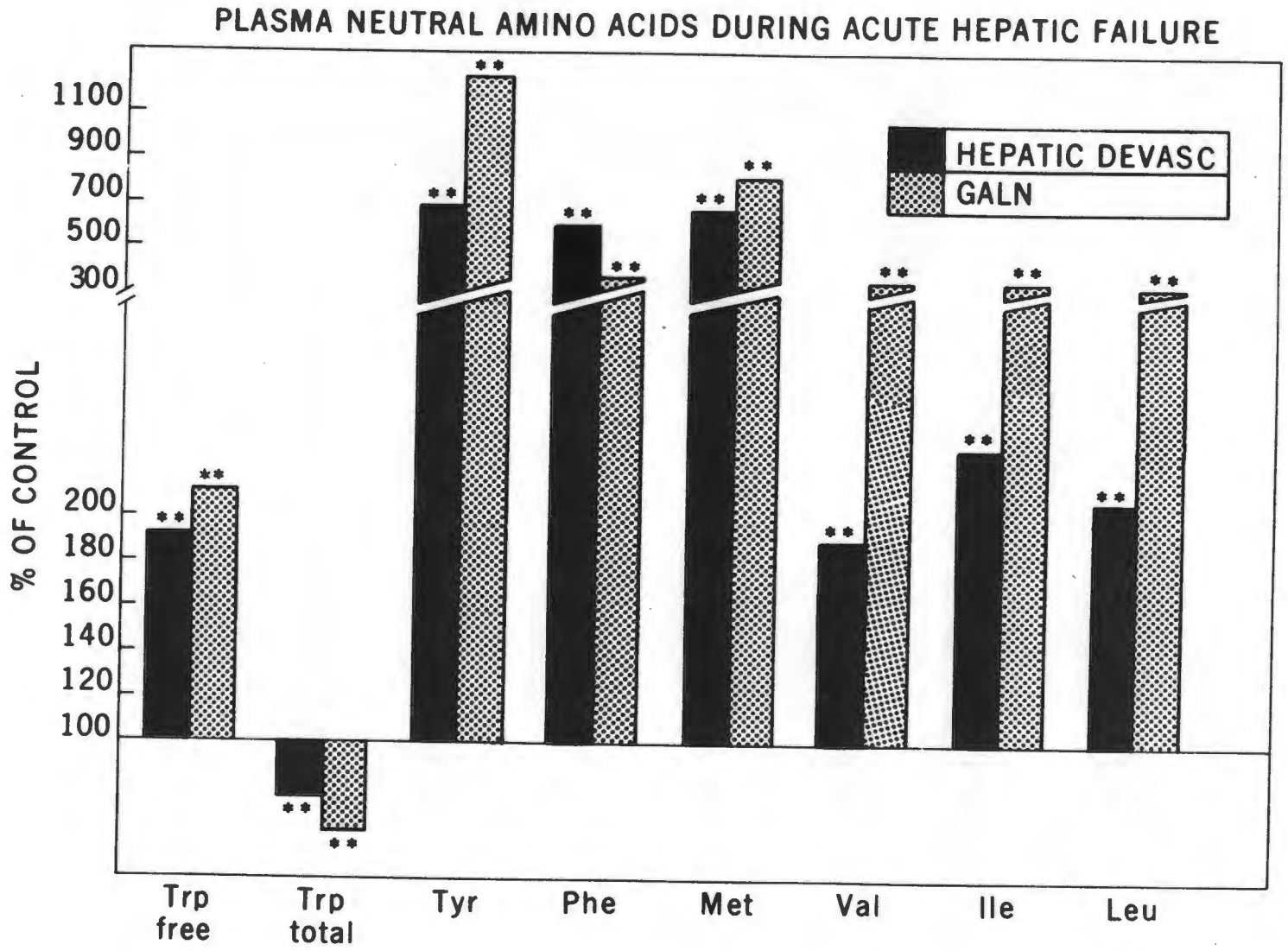

PLASMA NEUTRAL AMINO ACIDS DURING ACUTE HEPATIC FAILURE
% OF CONTROL
1100
900
700
500
300
200
180
160
140
120
100
HEPATIC DEVASC
GALN
**
Trp free
Trp total
Tyr
Phe
Met
Val
Ile
Leu

TABLE 17

PLASMA INSULIN AND GLUCAGON DURING ACUTE HEPATIC FAILURE

Refer to Tables 12 and 13 for experimental details. The results are expressed in ng/ml as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from control values are indicated by *p<0.001.

	Control	Hep. devasc.	GalN
Insulin	1.3 ± 0.3 (9)	1.8 ± 0.2 (9)	0.45 ± 0.09 (5)
Glucagon	0.41 ± 0.19 (9)	2.5 ± 0.2 (9)*	1.7 ± 0.1 (3)*

alone in dogs (Soeters et al., 1976a). The plasma insulin/glucagon ratio was also decreased in patients with spontaneous or surgical portacaval shunting (Sherwin et al., 1974). Similarly, rats with portacaval shunts had lower plasma insulin and higher plasma glucagon concentrations than control rats (Rossouw et al., 1978). No information seems to be available about changes in these hormones during acute hepatic failure.

DISCUSSION

The high concentrations of the plasma amino acids found during acute hepatic failure in patients have been attributed to release from the necrotic liver as well as catabolism in peripheral muscle. The striking rise of methionine, phenylalanine and tyrosine suggested a hepatic origin. The large increases in plasma amino acids observed after hepatectomy in the dog and rat (McMenamy et al., 1965; Tyce et al., 1967) indicate that voluntary muscle may contribute to these increases during acute hepatic failure. In the present study, the amino acid profile, including the 10-fold increase in alanine, as well as the decreased insulin/glucagon ratio, suggested a catabolic state. Inability of the liver to extract alanine would further increase its plasma concentrations. (Increased branched chain amino acids are also found in other clinical conditions associated with increased protein catabolism [Felig, 1975; Ruderman, 1975]). Since the liver is not being perfused by either the portal vein or the hepatic artery after the devascularization procedure, in this model the liver cannot be releasing large quantities of amino acids. The larger increases

observed in plasma amino acids during galactosamine-induced necrosis may be due to additional free amino acids originating from the liver.

The high unbound tryptophan concentrations found in the plasma are characteristic of acute hepatic failure. These may be caused by the high plasma free fatty acids found during hepatic failure (Ono et al., 1978) which release tryptophan from albumin, and low albumin concentrations (James et al., 1976; Ono et al., 1978) due to decreased synthesis by the failing liver. The increased unbound tryptophan could possibly lead to increased uptake by various tissues thus causing decreased concentrations of total tryptophan in the plasma.

Only small changes in plasma amino acids were observed during chronic hepatic failure. Others have suggested that the decreased branched chain amino acids during this condition are caused by increased uptake by muscle, which is largely responsible for their metabolism (Ruderman, 1975), and possibly by adipose tissue where increased incorporation into fatty acids may occur (Soeters & Fischer, 1976).

The possible effects of the plasma amino acid changes on brain amino acids is discussed in part 3 of this section.

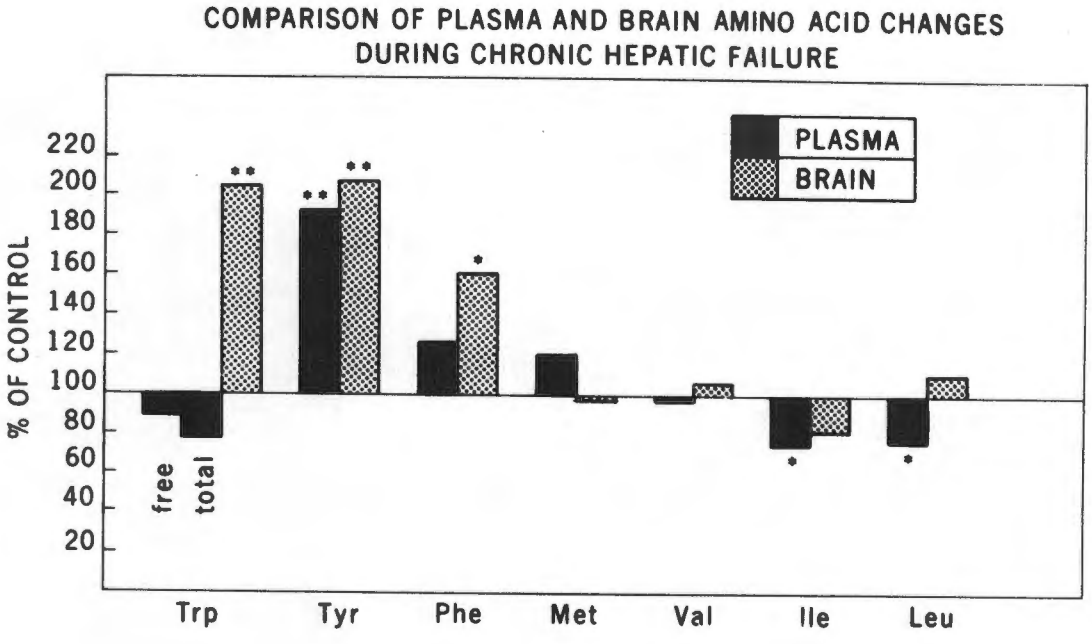
3. PLASMA/BRAIN AMINO ACID RELATIONSHIPS

Figures 11 and 12 show the changes in plasma and brain amino acids as compared to control values, during chronic and acute hepatic failure, respectively. The pattern in the two models of acute hepatic failure is very similar. Increases in plasma aromatic amino acids (including unbound but not total tryptophan) were associated with increases in brain concentrations. Plasma methionine and branched-chain amino acid increases on the other hand, were not accompanied by significant brain increases with the exception of leucine and methionine during galN hepatic failure. Thus these essential neutral amino acids competing for one transport mechanism are divided into two groups, those associated with neurotransmitter metabolism (the aromatic amino acids), which showed parallel increases in plasma and brain, and the others which are not involved in neurotransmitter metabolism and increased largely in the plasma.

During chronic hepatic failure, plasma and brain changes were much smaller. However, a parallel change in plasma and brain was seen in the case of tyrosine.

The ratio of unbound plasma tryptophan to brain tryptophan was similar in acute hepatic failure rats and controls. This finding, together with the significant decrease in total plasma tryptophan suggested that unbound tryptophan was a more important factor in determining brain concentrations than total tryptophan in these rats. The influence of plasma amino acids on brain tryptophan was further examined by testing for correlation between brain tryptophan and plasma unbound and total tryptophan, as well as ratios of unbound and

FIGURE 11



All rats were sampled four weeks after portacaval shunt or sham-operation (controls). The results are expressed as percentage of control, as shown by the bars. Results significantly different from control values are indicated by *$p<0.05$ and **$p<0.01$.

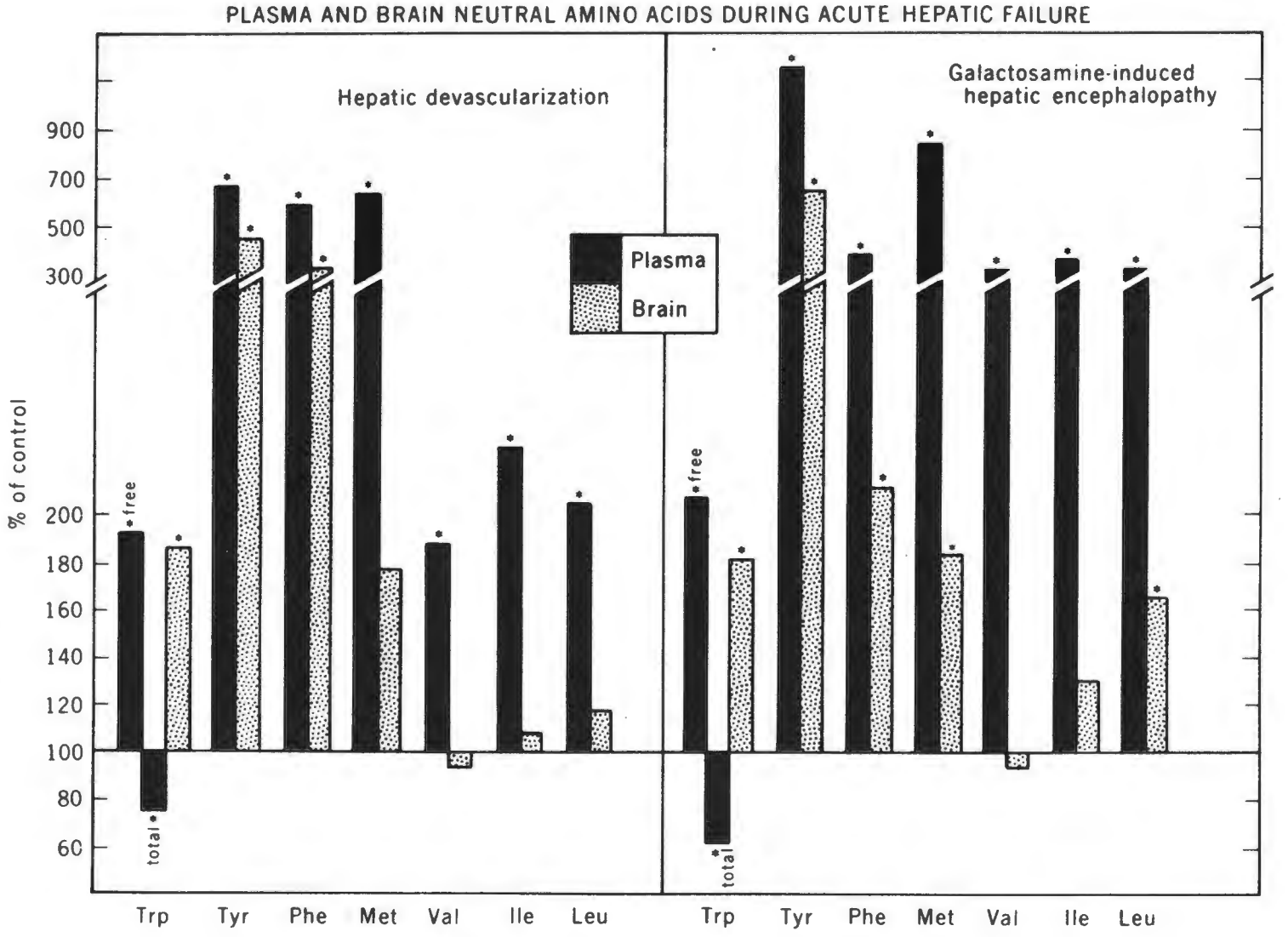
For legend to FIGURE 12, see over.

FIGURE 12

PLASMA AND BRAIN NEUTRAL AMINO ACIDS DURING ACUTE HEPATIC FAILURE

Acute hepatic failure was produced either by hepatic devascularization or with galactosamine. (See Figures 6 and 9 for experimental details.) The bars represent percentage of control values with significantly different results indicated by *$p<0.01$.

FIGURE 12


PLASMA AND BRAIN NEUTRAL AMINO ACIDS DURING ACUTE HEPATIC FAILURE
Hepatic devascularization
Galactosamine-induced hepatic encephalopathy
Plasma
Brain
% of control
900
700
500
300
200
180
160
140
120
100
80
60
free
total
Trp
Tyr
Phe
Met
Val
Ile
Leu

total tryptophan to the competing amino acids tyrosine, phenylalanine, methionine, leucine, isoleucine, and valine, i.e.

(a) Brain tryptophan vs. unbound tryptophan

(b) Brain tryptophan vs. total tryptophan

(c) Brain tryptophan vs. unbound tryptophan ÷ sum competitors

(d) Brain tryptophan vs. total tryptophan ÷ sum competitors (Figure 13 and 14).

The values of the sums of the competitors and ratios in the various groups are given in Table 18. The sum of competitors increased in all conditions, including chronic hepatic failure. Due to the large increase in competing amino acids the unbound tryptophan ÷ sum ratio was significantly decreased during acute hepatic failure, in spite of the increase in unbound tryptophan. The total tryptophan ÷ sum ratio, as expected from the lowered total tryptophan, showed a large decrease.

No significant correlations were obtained during chronic hepatic failure, when testing either shunted or control groups or both groups together (Figure 13).

During acute hepatic failure (Figure 14) there was a significant positive correlation between brain tryptophan and unbound tryptophan (Figure 14A) and a negative correlation between brain tryptophan and plasma total tryptophan (Figure 14B) when all rats were examined together. Similarly, a negative correlation was obtained between brain tryptophan and the total tryptophan to sum of competitors ratio (Figure 14D). There was no significant correlation between brain tryptophan and the unbound tryptophan to sum of competitors ratio (Figure 14C).

TABLE 18

BRAIN TRYPTOPHAN, PLASMA TRYPTOPHAN AND THE RATIO OF PLASMA TRYPTOPHAN TO THE SUM OF ITS COMPETITORS, DURING HEPATIC FAILURE

See Tables 11, 12 and 13 for experimental details. The results are given in μmol/g or μmol/ml as mean ± S.E.M. with the number of rats in each group in parenthesis. Results significantly different from control are indicated by *p<0.05; **p<0.001.

	Chronic Hepatic Failure		Acute Hepatic Failure		
	Control (6)	Pcs (7)	Control (13)	Hep. devasc. (14)	GalN (10)
Brain tryptophan	0.020 ± 0.008	0.047 ± 0.001**	0.029 ± 0.002	0.053 ± 0.003**	0.044 ± 0.003**
Plasma unbound tryptophan	0.016 ± 0.005	0.018 ± 0.003	0.018 ± 0.002	0.033 ± 0.003**	0.037 ± 0.006**
Plasma total tryptophan	0.182 ± 0.021	0.191 ± 0.009	0.220 ± 0.001	0.159 ± 0.006**	0.141 ± 0.009**
Sum of competitors (tyr + phe + met + ile + leu + val)	0.54 ± 0.03	0.59 ± 0.04	0.53 ± 0.04	1.8 ± 0.2**	3.1 ± 0.4**
$\frac{\text{Unbound tryptophan}}{\text{sum}}$	0.030 ± 0.012	0.031 ± 0.005	0.034 ± 0.008	0.019 ± 0.003*	0.013 ± 0.003*
$\frac{\text{Total tryptophan}}{\text{sum}}$	0.34 ± 0.03	0.32 ± 0.01	0.42 ± 0.07	0.088 ± 0.007*	0.045 ± 0.007**

For legend to FIGURE 13, see over.

FIGURE 13

CORRELATION BETWEEN BRAIN TRYPTOPHAN AND VARIOUS PLASMA AMINO ACID PARAMETERS IN CHRONIC HEPATIC FAILURE

Samples were obtained four weeks after portacaval shunt (pcs) or sham operation (controls). For each rat, brain tryptophan concentration was compared to:

(A) the unbound plasma tryptophan concentration,

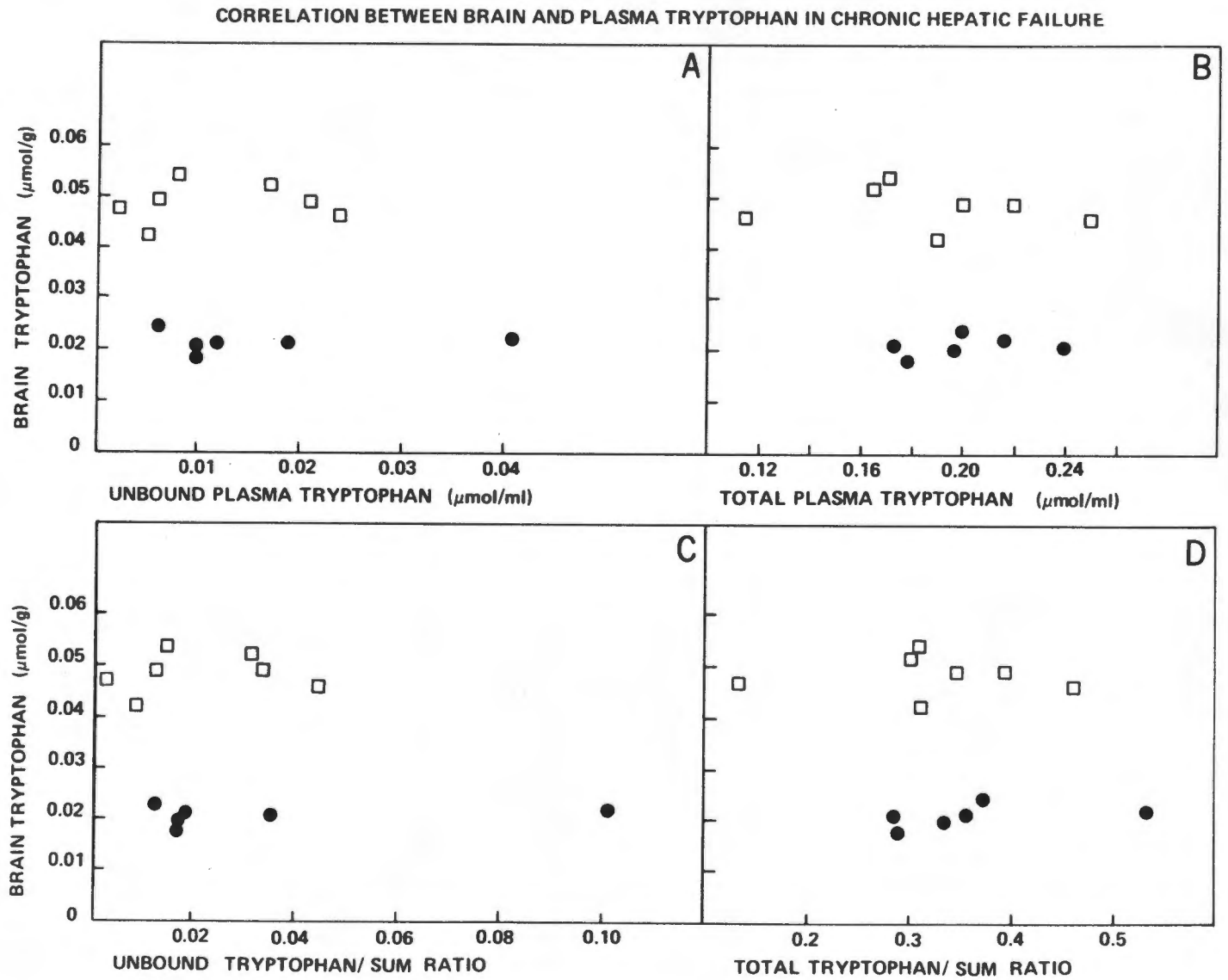
(B) the total plasma tryptophan concentration,

(C) the ratio of unbound plasma tryptophan to the sum of plasma tyrosine + phenylalanine + methionine + isoleucine + leucine + valine (unbound tryptophan/sum ratio),

(D) the ratio of total plasma tryptophan to the sum of amino acids as in (c).

The correlations were not significant for any of the comparisons. ● = control. □ = pcs.

FIGURE 13


CORRELATION BETWEEN BRAIN AND PLASMA TRYPTOPHAN IN CHRONIC HEPATIC FAILURE
A
B
C
D
BRAIN TRYPTOPHAN (μmol/g)
0.06
0.05
0.04
0.03
0.02
0.01
0
0.01
0.02
0.03
0.04
UNBOUND PLASMA TRYPTOPHAN (μmol/ml)
0.12
0.16
0.20
0.24
TOTAL PLASMA TRYPTOPHAN (μmol/ml)
0.02
0.04
0.06
0.08
0.10
UNBOUND TRYPTOPHAN/ SUM RATIO
0.2
0.3
0.4
0.5
TOTAL TRYPTOPHAN/ SUM RATIO

For legend to FIGURE 14, see over.

FIGURE 14

CORRELATION BETWEEN BRAIN TRYPTOPHAN AND VARIOUS AMINO ACID PARAMETERS IN ACUTE HEPATIC FAILURE

Acute hepatic failure was produced either by hepatic devascularization or with galactosamine. (For experimental details see Figures 6 and 9.) For each rat, brain tryptophan content was compared to:

(A) the unbound plasma tryptophan concentration,

(B) the total plasma tryptophan concentration,

(C) the ratio of unbound plasma tryptophan to the sum of the competing amino acids as given in Figure 13,

(D) the ratio of total plasma tryptophan to the sum of the competing amino acids.

● = control. □ = hepatic devascularization. Δ = galactosamine-induced hepatic failure.

The correlation coefficients were as follows:

(A) $r = +0.53$ ($p<0.001$ that $r = 0$)

(B) $r = -0.34$ ($p<0.05$ that $r = 0$)

(C) $r = -0.24$ (not significant)

(D) $r = -0.54$ ($p<0.001$ that $r = 0$)

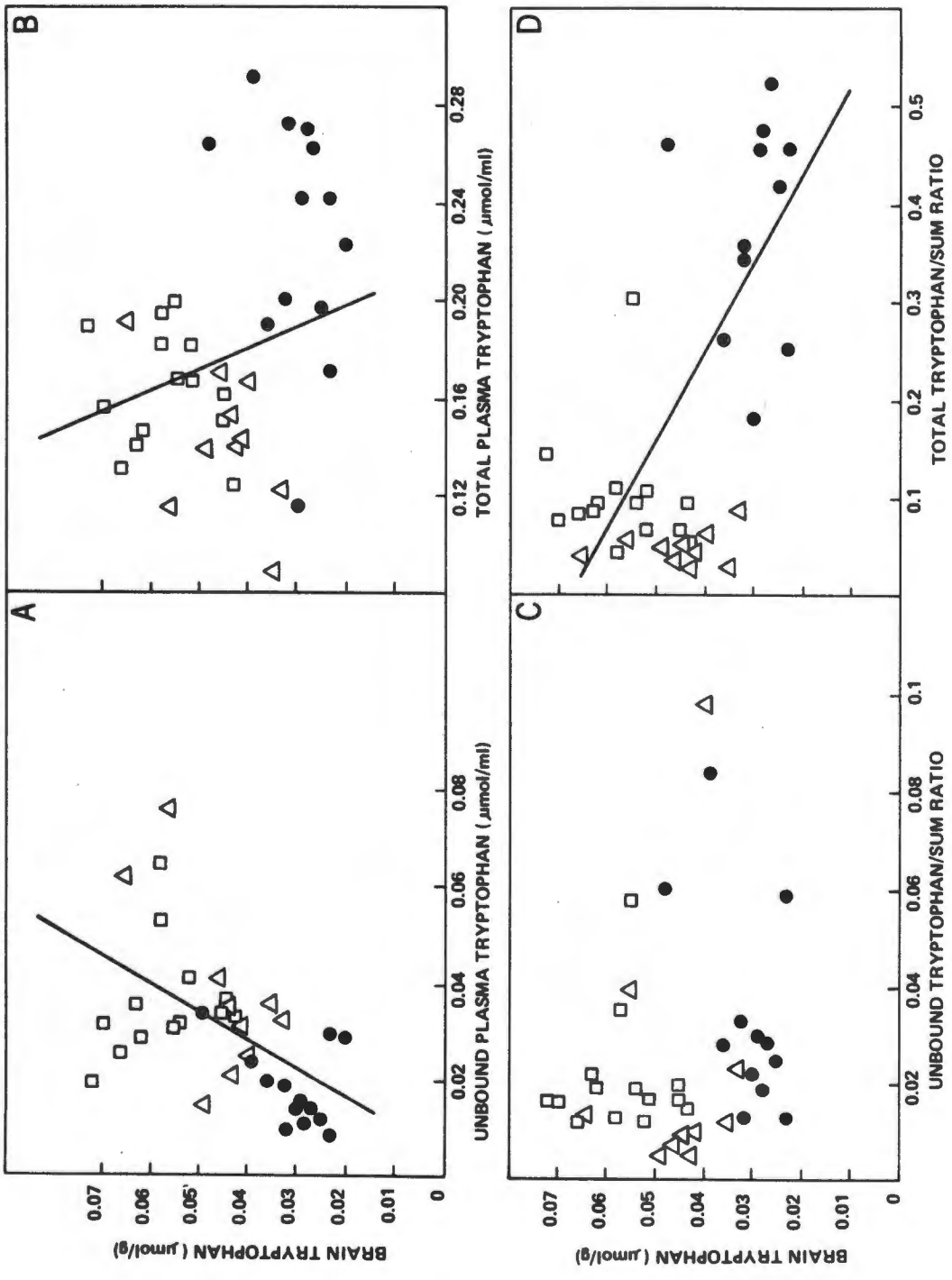
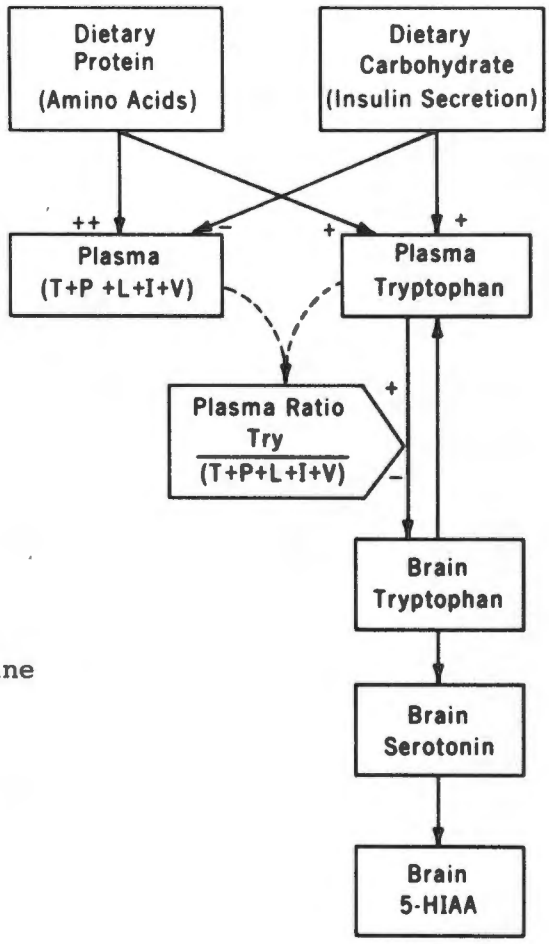

CORRELATION BETWEEN BRAIN AND PLASMA TRYPTOPHAN IN ACUTE HEPATIC FAILURE
A
B
C
D
BRAIN TRYPTOPHAN (μmol/g)
UNBOUND PLASMA TRYPTOPHAN (μmol/ml)
TOTAL PLASMA TRYPTOPHAN (μmol/ml)
UNBOUND TRYPTOPHAN/SUM RATIO
TOTAL TRYPTOPHAN/SUM RATIO
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.02
0.04
0.06
0.08
0.1
0.12
0.16
0.20
0.24
0.28
0.1
0.2
0.3
0.4
0.5

FIGURE 14

These results contrast with those of Fernstrom, Hirsch & Faller (1976) who found that in normal rats correlations involving total tryptophan were more positive than those involving unbound tryptophan. This discrepancy suggests that extrapolations from normal to pathological conditions, as have been made to account for increased brain tryptophan in hepatic encephalopathy, must be made with caution, since new variables may be present.

DISCUSSION

Tryptophan plays a unique role in amino acid metabolism. It is the least abundant amino acid in mammals and may be a limiting factor in protein synthesis, especially in young animals (Munro, 1974). Tryptophan is also the only plasma amino acid which is bound to albumin (McMenamy, Lund & Oncley, 1957; McArthur & Dawkins, 1969). Much work has been done to study the regulation of tryptophan uptake into the brain. The two main factors involved are firstly, competition with other neutral amino acids for the same uptake carrier mechanism, and secondly, the proportion of tryptophan bound to plasma albumin. The effect of competing amino acids on tryptophan transport into the brain has been extensively studied in normal rats in experiments where fluctuations in plasma amino acids remained within physiological limits (Fernstrom & Wurtman, 1971b, 1972; Fernstrom et al., 1976; Colmenares, Wurtman & Fernstrom, 1975; Pérez-Cruet, Chase & Murphy, 1974). When a diet consisting only of carbohydrate or fat and/or protein lacking the amino acids phenylalanine, tyrosine, leucine, isoleucine, and valine was fed to rats, a rise in brain tryptophan was obtained (Fernstrom & Wurtman, 1972; Fernstrom et al., 1976).



T = tyrosine

P = phenylalanine

L = leucine

I = isoleucine

V = valine

However, if normal protein was included no rise was seen. This was explained as being due to the relatively low proportion of tryptophan in normal dietary protein. After a protein meal the competing amino acids in the plasma show a greater rise than tryptophan and thus there is no increase in tryptophan uptake. These diet-induced changes in brain tryptophan and hence serotonin concentrations were summarized as shown in the diagram above (from Fernstrom & Wurtman, 1972). Similar results were obtained when feeding various amino

acid mixtures to rats (Gessa, Biggio, Fadda, Corsini & Tagliamonte, 1974). If only phenylalanine, tyrosine, leucine, isoleucine, methionine, histidine or all the essential amino acids excluding tryptophan were fed, there was a decrease in brain tryptophan. If a mixture of amino acids without those listed was given, no decrease was observed.

Injection of neutral amino acids (histidine, isoleucine, leucine, methionine, phenylalanine, tyrosine, valine) in the normal physiological range was shown to decrease brain uptake of tryptophan (Etienne et al., 1976). These experiments are strong evidence that competition plays an important role in the regulation of tryptophan uptake in normal rats.

The second factor which is thought to influence this process is the extent to which tryptophan is bound to albumin in the plasma. Unbound tryptophan has been found to influence brain tryptophan in many circumstances, for example:

(a) after food intake or insulin injection (Pérez-Cruet et al., 1974; Curzon & Knott, 1974b),

(b) during food deprivation (Knott, Joseph & Curzon, 1973),

(c) during immobilization stress (Knott et al., 1973),

(d) during treatment with drugs affecting tryptophan binding (Guerinot et al., 1974; Tagliamonte, Biggio, Vargiu & Gessa, 1973a; Gessa et al., 1974),

(e) in early life (Bourgoin, Faivre-Bauman, Benda, Glowinski & Hamon, 1974),

(f) in acute experimental hepatic failure in pigs (Curzon et al., 1973b; Buxton et al., 1974).

However, increases in plasma unbound tryptophan do not always lead to increased tryptophan in the brain (Madras et al., 1973, 1974). In addition, food intake leads to an increase in brain tryptophan even though the resulting insulin secretion causes decreased plasma unbound tryptophan levels (by lowering free fatty acids which release tryptophan from its binding sites on albumin) (Madras et al., 1973, 1974; Curzon, Friedel & Knott, 1973a; Curzon et al., 1974; Curzon & Knott, 1974a). Some of the conditions listed may also be associated with changes in the competing amino acids, for example fenfluramine and probenecid, as well as increasing plasma unbound tryptophan, cause a decrease in plasma tyrosine (Tagliamonte et al., 1971). This matter is further complicated by the various methods used to separate unbound from bound tryptophan. This must be done under carefully controlled conditions since factors such as pH, temperature and the presence of heparin may affect the binding constant. For example, it has been suggested that in vitro lipolysis in serum after collection, can lead to high unbound tryptophan levels which would not be indicative of the true in vivo situation (Hutson et al., 1976; see also Fernstrom, Munro & Wurtman, 1977).

Significant correlations have been obtained between brain tryptophan content and

(a) plasma total tryptophan concentrations (Fernstrom et al., 1976),

(b) plasma unbound tryptophan concentrations (Knott & Curzon, 1972; Tagliamonte, Biggio, Vargiu & Gessa, 1973b),

(c) the ratio of plasma total tryptophan to the sum of the competing amino acids (Fernstrom et al., 1976),

(d) the ratio of plasma unbound tryptophan to the sum of the competing amino acids (Pérez-Cruet et al., 1974).

The relative importance of the effects of competition by other amino acids and unbound tryptophan concentrations in regulating normal brain tryptophan transport is controversial at present, but it seems that both these factors play a role in the process, their individual importance depending on specific conditions. Dynamic factors, such as rapid re-equilibrium between free and unbound tryptophan in the brain, may alter the relative importance of the factors influencing brain uptake, during passage of blood through the brain.

Much attention has been paid to tryptophan transport and its regulation in the investigation of chronic and acute hepatic failure, because brain tryptophan is thought to be involved in the aetiology of hepatic encephalopathy. Hypotheses have been put forward implicating plasma amino acid changes in the increase in brain tryptophan content (Munro et al., 1975; Soeters & Fischer, 1976). The results in the present study indicated that during hepatic failure, plasma competing amino acid and unbound tryptophan changes do not explain the increases in brain tryptophan observed, and additional factors must be considered. During chronic hepatic failure, changes in the plasma concentrations of the competing amino acids (an increase in tyrosine and decreases in leucine and isoleucine) resulted in no significant alteration in the total molar sum of the competing amino acids (portacaval shunt: 0.59 ± 0.04, control: 0.54 ± 0.03, Table 18) due to the large increase in tyrosine and small decreases in the other two amino acids. Thus it seemed very unlikely that these changes resulted in reduced competition for tryptophan uptake and

thus caused the approximate doubling of brain tryptophan content observed. Therefore, these experiments did not support the hypothesis that altered plasma amino acid patterns lead to increased brain tryptophan by lowering competition for transport during chronic hepatic failure (Munro et al., 1975; Soeters & Fischer, 1976). Nor could the increase in brain tryptophan be attributed to any change in plasma tryptophan, since both unbound and total tryptophan were unchanged and no correlation was obtained between plasma and brain changes. Thus, at this stage, there seemed to be no explanation for the high brain tryptophan content without considering possibilities of decreased utilization by and removal from the brain.

As indicated by a few earlier studies of acute hepatic failure, the picture presented by the plasma amino acid concentrations, with several-fold increases in all amino acids except tryptophan, was very different from that found during the chronic condition. It is clear that the altered plasma amino acids could not have been responsible for the increased brain tryptophan; in fact, if dependent only on competition effects, brain tryptophan should have been decreased. Unbound plasma tryptophan showed a similar increase to brain tryptophan which suggests that it had a greater influence. However, whether the increase in unbound tryptophan was sufficient to overcome the effect of the very high competing amino acid concentrations (it seemed unlikely from earlier evidence, that these would have no effect at all) remained an open question.

Therefore, as with chronic hepatic failure, changes in plasma amino acids could not satisfactorily account for the increased brain tryptophan content. This finding directed attention to another aspect

of tryptophan transport, namely the uptake mechanism itself. Studies showing competition between the neutral amino acids for transport across the blood-brain barrier were all performed in normal rats (Fernstrom et al., 1976; Etienne et al., 1976; Oldendorf & Szabo, 1976) and the hypotheses attempting to explain brain tryptophan changes in terms of plasma amino acid patterns were based on these findings. However, it had not been established that the carrier mechanisms remained unaltered during pathological conditions such as hepatic failure.

In the light of the above results, it seemed possible that during chronic and acute hepatic failure the transport system was altered, resulting in different uptake patterns from normal. For example, the affinities of the transport carrier for various substrates such as tryptophan might have been increased, or the blood-brain barrier might have become generally more permeable. The experiments investigating these possibilities are described in Results Section III.

4. EFFECTS OF INTRAVENOUS INFUSION OF AMINO ACIDS ON BRAIN AND PLASMA METABOLITES

Intravenous infusion of special amino acid mixtures to dogs with portacaval shunts and patients with chronic hepatic failure has been shown to have beneficial effects in several studies (Fischer et al., 1975; Soeters, Ebeid, Hodgman, James, Yoshimura, Westman & Fischer, 1976b; Fischer et al., 1976). These amino acid mixtures were specially formulated to correct the abnormal plasma amino acid pattern found in chronic hepatic failure, thus possibly influencing brain uptake of neurotransmitter precursors. A further stimulus to attempts to formulate specific amino acid mixtures for intravenous use in hepatic failure was the well-known intolerance of protein by patients with severe liver disease, especially if given orally. For example, a milk diet is tolerated better by these patients than a similar amount of protein given as meat which has a higher content of aromatic amino acids phenylalanine, tyrosine and tryptophan (Condon, 1971). Similarly, the infusion of various amino acid mixtures in amounts sufficient to provide adequate nutrition in hepatic failure results in a worsening of encephalopathy associated with a markedly abnormal plasma amino acid pattern (Fischer et al., 1974). In dogs with portacaval shunts, survival could also be related to various diets, the shortest survival correlating with a greater dietary intake of aromatic amino acids (Condon, 1971).

In the experiments by Fischer et al. (1975) and Soeters et al. (1976b), dogs with portacaval shunts, showing symptoms of hepatic encephalopathy, were given various intravenous amino acid mixtures. While administration of a commercially available synthetic amino acid

mixture led to increased encephalopathy and high mortality, a specially formulated mixture was associated with 100% survival and improvement of mental status, as well as normalization of plasma amino acids. This mixture, F080, contained reduced concentrations of aromatic and increased concentrations of branched chain amino acids, compared to the commercially available mixtures.

Patients with chronic hepatic failure showed a similar favourable response to F080 administration, associated with decreased plasma aromatic and increased branched chain amino acid levels (Fischer et al., 1976).

In these studies the normalization of plasma amino acids and improvement in mental status was attributed to the amino acids and their proportions in the solution given. However, a control solution containing all the ingredients but no amino acids was not used.

a. ACUTE HEPATIC FAILURE

Preliminary experiments, in which rats were infused with the amino acid solution F080 after hepatic devascularization, showed no improvement in brain or plasma metabolites, and in fact shortened the survival time compared to untreated rats. In view of the very high plasma amino acid concentrations, including the branched chain amino acids, found in the rats with acute hepatic failure, these results were not unexpected. Therefore these experiments were not taken further in the acute hepatic failure condition.

b. CHRONIC HEPATIC FAILURE

The previously reported work on the effects of intravenous amino acids in hepatic failure was carried out in dogs with portacaval shunts and patients with chronic hepatic failure. No similar work had been done with rats with portacaval shunts. Therefore, even though the plasma amino acid changes found in the present study could not account for the brain amino acid abnormalities (as described in part 3 of this section), it was thought that the effects of continuous intravenous infusion of amino acids might be of interest, not only as a possible therapeutic measure but also to shed more light on plasma/brain amino acid relationships in chronic hepatic failure.

This section describes investigations of plasma and brain metabolite changes after continuous intravenous infusion of solutions with and without amino acids, to rats four weeks after portacaval shunting.

PROCEDURE

Portacaval shunts and sham-operations were performed as described in the Experimental Section. Rats were fed intravenously for four days before sampling at four weeks after portacaval shunt or sham-operation. The intravenous solutions were administered to unrestrained rats according to the procedure of Steiger, Vars & Dudrick (1972). Using halothane as anaesthetic (1.5% in oxygen), a catheter (19 gauge needle, Deseret Intracath) was inserted into the jugular vein over the clavicle, advanced into the superior vena cava as far as the right atrium and secured with two silk sutures. The free end of the catheter was passed subcutaneously to exit through the skin of the midscapular area where it was held in position by a specially constructed harness.

The intravenous catheter was connected by means of Portex plastic tubing to a sterile swivel infusion apparatus (Model 192-03 Cannula Feed-through Swivel, Lehigh Valley Electronics Inc., Fogelsville, Pennsylvania, USA) which allowed the animal complete freedom of movement.

The intravenous solution was infused from a glass syringe at a continuous rate of about 35 ml per 24 hours by a constant infusion pump (B. Braun Melsungen) fixed above the cage. No additional food or water was given.

This technique allowed total parenteral nutrition for prolonged periods. Preliminary experiments with normal rats showed that it was well tolerated for at least five days. The composition of the solutions used is given in Table 19.

RESULTS

Intravenous feeding of amino acids and dextrose or dextrose alone resulted in a striking return towards normal of nearly all the brain metabolites that were altered in the rat four weeks after portacaval shunt (Table 20). After F080 plus dextrose infusion, ammonia concentrations, although reduced, were still significantly greater than normal, while aspartate, which was decreased in untreated portacaval shunted rats, was even further decreased by F080 + dextrose (Figure 15). Dextrose alone had a greater effect on ammonia and glutamine, and raised aspartate, so that all these metabolites were statistically in the normal range in this group of rats.

Similar effects were seen on the brain neutral amino acids (Table 20, Figure 16). The high tryptophan, tyrosine and phenylalanine were

TABLE 19

COMPOSITION OF SOLUTIONS USED FOR INTRAVENOUS INFUSION

	Solution	
Amino Acids (µmol/ml)	*F080 + dextrose	dextrose alone
L-tryptophan	1.86	-
L-phenylalanine	3.03	-
L-methionine	3.35	-
L-isoleucine	34.31	-
L-leucine	41.93	-
L-valine	35.85	-
L-lysine-acetate	16.12	-
L-alanine	42.09	-
L-arginine	17.22	-
L-histidine	7.73	-
L-proline	34.74	-
L-serine	23.79	-
Glycine	59.94	-
L-cysteine-HCl-H_2O	0.57	-
Electrolytes (mEq/1000 ml)		
Na^+	130	130
K^+	4	4
Mg^{2+}	3	3
HCO_3^-	28	28
Cl^-	109	109
Dextrose (g/100 ml)	5	5

* F080 is an experimental amino acid solution designed for hepatic failure by McGaw Laboratories, Glendale, California, U.S.A.

TABLE 20

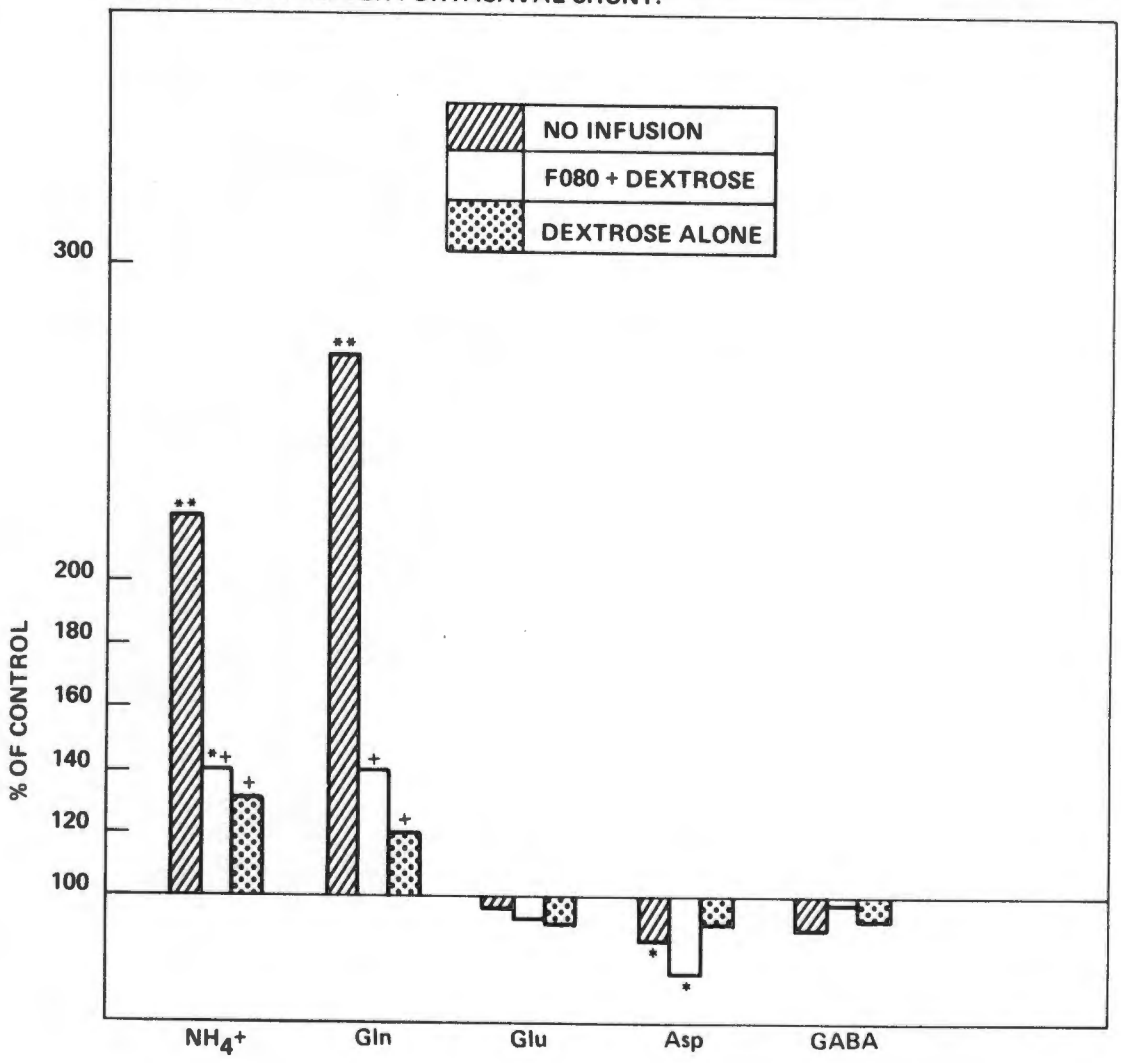
THE EFFECT OF INTRAVENOUS FEEDING ON BRAIN METABOLITES FOUR WEEKS AFTER PORTACAVAL SHUNT

All rats were sampled four weeks after portacaval shunt (pcs) or sham operation (control). IV-infused rats were infused for four days (35 ml solution per 24 h) before sampling. The composition of the solutions is given in Table 19. The results are expressed in µmol/g wet weight tissue as mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from untreated control are indicated by *p<0.05, **p<0.001; those different from untreated portacaval shunt by +p<0.01.

	Untreated						IV-Infused					
Ammonia and Metabolites	Control			Pcs			Pcs + F080 + dext.			Pcs + dext.		
NH_4^+	0.40	± 0.04	(7)	0.97	± 0.10	(9)**	0.56	± 0.07	(6)*+	0.54	± 0.08	(11)+
Glutamine	5.85	± 0.30	(11)	15.9	± 1.0	(13)**	8.33	± 0.96	(6)+	6.99	± 0.09	(11)+
Glutamate	10.5	± 0.4	(11)	10.1	± 0.4	(13)	9.70	± 0.97	(6)	9.73	± 0.38	(11)
Aspartate	2.50	± 0.08	(5)	2.16	± 0.07	(4)*	2.00	± 0.09	(3)*	2.29	± 0.19	(4)
GABA	2.6	± 0.1	(11)	2.3	± 0.1	(10)	2.5	± 0.3	(6)	2.4	± 0.2	(10)
Neutral Amino Acids												
Tryptophan	0.024	± 0.002	(11)	0.051	± 0.003	(13)**	0.023	± 0.002	(6)+	0.028	± 0.002	(11)+
Tyrosine	0.084	± 0.009	(11)	0.17	± 0.02	(8)**	0.15	± 0.02	(4)*	0.094	± 0.010	(10)+
Phenylalanine	0.097	± 0.020	(11)	0.16	± 0.02	(8)*	0.085	± 0.009	(4)+	0.088	± 0.009	(10)+
Methionine	0.054	± 0.012	(7)	0.053	± 0.005	(7)	0.014	± 0.001	(2)+	0.035	± 0.008	(8)+
Valine	0.092	± 0.011	(10)	0.098	± 0.010	(6)	0.26	± 0.02	(6)+	0.15	± 0.02	(10)
Leucine	0.11	± 0.01	(11)	0.12	± 0.01	(9)	0.19	± 0.02	(6)*+	0.13	± 0.01	(10)
Isoleucine	0.060	± 0.010	(11)	0.047	± 0.002	(9)	0.10	± 0.01	(6)*+	0.061	± 0.011	(10)+

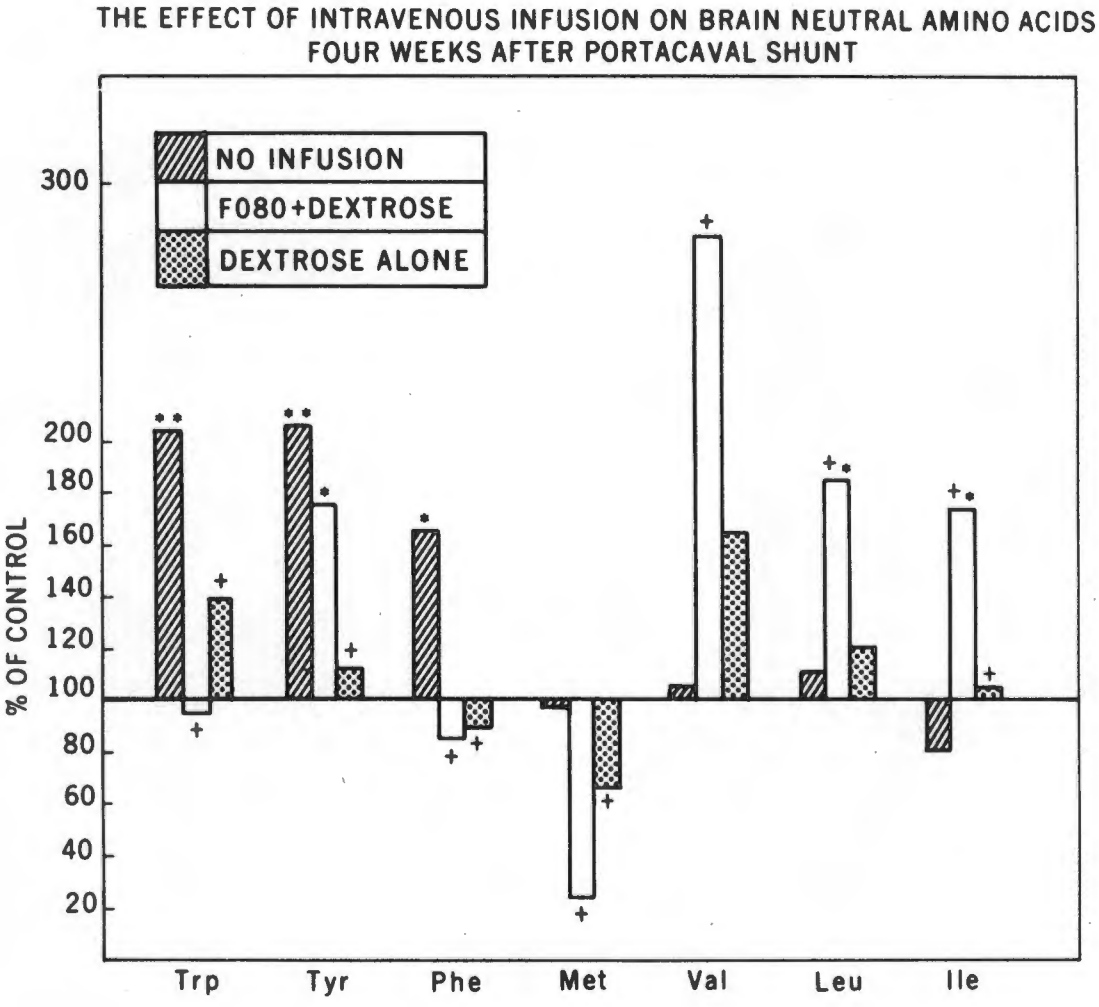
FIGURE 15

THE EFFECT OF INTRAVENOUS INFUSION ON BRAIN AMMONIA AND METABOLITES FOUR WEEKS AFTER PORTACAVAL SHUNT.



All rats were sampled four weeks after portacaval shunt (pcs) or sham operation. Three groups of rats are shown: pcs fed normally, pcs infused with F080 + dextrose solution, and pcs infused with dextrose only solution (see Table 19 for composition of solutions). The infused rats were treated for four days before sampling (see Procedure section for further details). The results are shown as percentage of control (normally fed sham-operated rats) as indicated by the bars. Results significantly different from control values are indicated by *$p<0.05$, **$p<0.001$, and those different from untreated rats by $^{+}p<0.01$.

FIGURE 16



See Figure 15 for details. The bars indicate percentage of control values. Results significantly different from control are indicated by *$p<0.005$, **$p<0.001$, and those different from untreated rats by $^{+}p<0.01$.

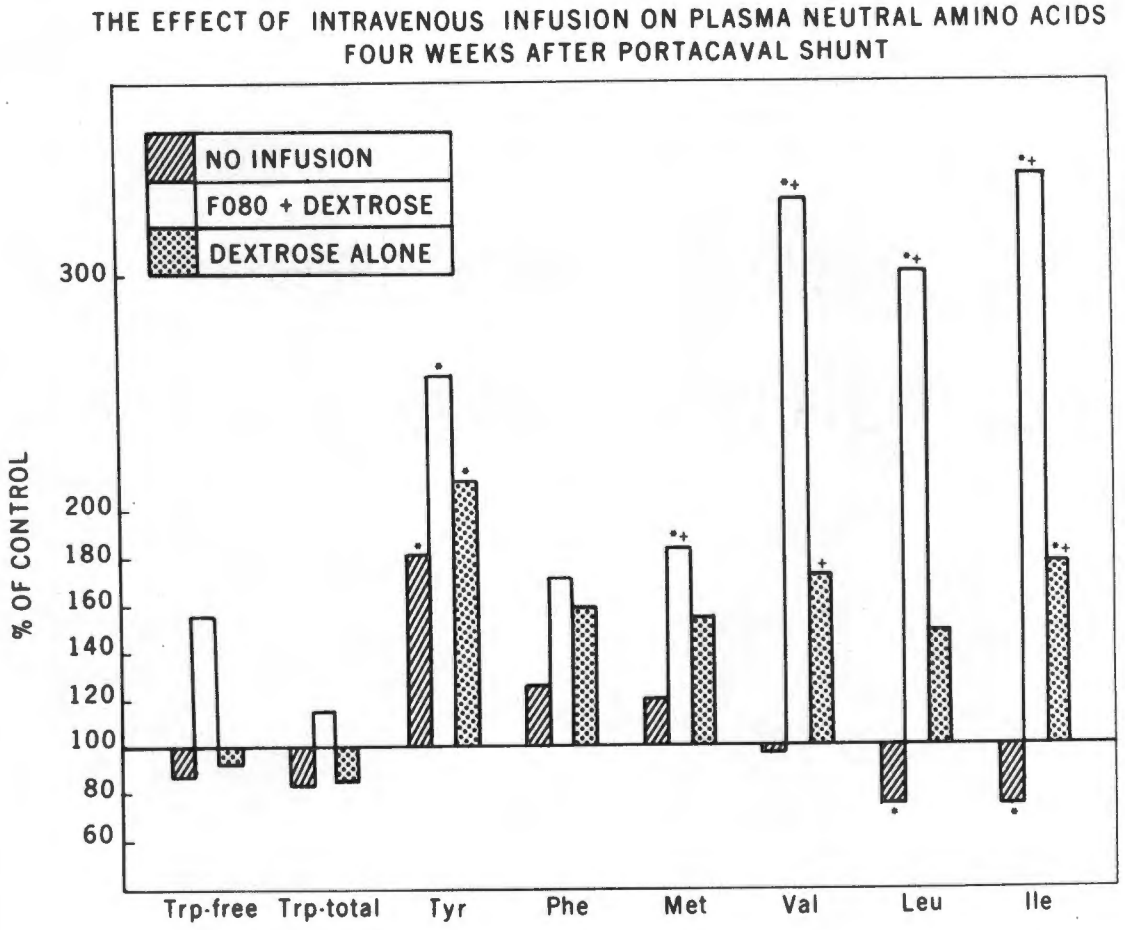
normalized by the infusion although tyrosine was still significantly greater than normal after F080 + dextrose. This solution also increased brain isoleucine and leucine, possibly the result of the high plasma concentrations of these amino acids. All the amino acids were in the normal range after infusion of dextrose alone.

The effect of IV infusions on plasma amino acids is shown in Figure 17, while absolute values are given in Table 21. The two solutions had essentially similar effects in that the reduced branched chain amino acids were increased to normal or higher (with F080 + dextrose) while the other amino acid altered after portacaval shunt, tyrosine, was not normalized and remained significantly higher than in control rats. F080 + dextrose infusion caused very high branched chain amino acid concentrations (200 to 240% of normal) which was to be expected from the amounts of these amino acids in the solution; however, infusion of dextrose alone also normalized these amino acids and even caused significantly higher isoleucine concentrations when compared to control rats. Methionine was increased by infusion of F080 + dextrose while alanine was increased by both solutions.

DISCUSSION

The above experiments show a striking return to normal of most brain metabolites after infusion of either amino acids plus dextrose or dextrose alone. This was accompanied by normalization of the lowered plasma leucine and isoleucine. The finding that the dextrose solution was at least as effective as the amino acid combination in correcting brain metabolite abnormalities suggested that factors other than the presence or proportions of neutral amino acids in the intravenous

FIGURE 17



See Figure 15 for details. The bars indicate percentage of control. Results significantly different from control values are indicated by *$p<0.02$ and those different from untreated rats by $^{+}p<0.05$.

TABLE 21

THE EFFECT OF INTRAVENOUS FEEDING ON PLASMA AMINO ACIDS FOUR WEEKS AFTER PORTACAVAL SHUNT

See Table 20 for details. The results are expressed in μmol/ml as mean ± S.E.M. with the numbers of rats in parenthesis. Results significantly different from untreated control are indicated by *p<0.02; those different from untreated portacaval shunt by +p<0.05.

	Untreated		IV-Infused	
	Control (12)	Pcs (12)	pcs + F080 + dext. (6)	Pcs + dext. (12)
Tryptophan - unbound	0.012 ± 0.002	0.011 ± 0.003	0.018 ± 0.005	0.011 ± 0.002
Tryptophan - total	0.20 ± 0.01	0.18 ± 0.02	0.24 ± 0.03	0.18 ± 0.02
Tyrosine	0.051 ± 0.010	0.097 ± 0.010*	0.13 ± 0.05*	0.11 ± 0.02*
Phenylalanine	0.073 ± 0.010	0.091 ± 0.010	0.13 ± 0.03	0.12 ± 0.03
Methionine	0.047 ± 0.004	0.056 ± 0.011	0.088 ± 0.019*+	0.068 ± 0.009
Valine	0.17 ± 0.01	0.16 ± 0.02	0.53 ± 0.12*+	0.28 ± 0.06+
Leucine	0.15 ± 0.01	0.11 ± 0.01*	0.42 ± 0.08*+	0.21 ± 0.05
Isoleucine	0.069 ± 0.005	0.051 ± 0.005*	0.24 ± 0.04*+	0.13 ± 0.02*+
Glycine	0.34 ± 0.03	0.32 ± 0.03	0.58 ± 0.19	0.30 ± 0.06
Alanine	0.31 ± 0.03	0.40 ± 0.08	1.36 ± 0.53	0.57 ± 0.09*

solution were involved. Since these rats were not being fed orally, one possibility is that the total absence of protein intake (which has been associated with the development of encephalopathy in patients), results in normalization of brain metabolites. In the rats given F080 there would be a reduction of protein intake from about 2.4 g per day if normally fed, to about 1.3 g per day in the intravenous solution, and this may be sufficient to result in improvement. The exact mechanism remains an open question since, as previously discussed, the plasma amino acid abnormalities in rats with portacaval shunts are very small and in fact, the amino acid showing the greatest change from normal, tyrosine, was not normalized by intravenous infusion and therefore does not appear to be associated with the changes occurring in the brain.

Subsequent to carrying out the above experiments, similar work using F080 has been published (Rosen et al., 1978). In this study rats with portacaval shunts were used and the normalization of plasma amino acid abnormalities was accompanied by decreased brain tyrosine and phenylalanine although a relationship with brain tryptophan was not observed. However, as with the earlier experiments using dogs, no animals were given the infusion without the amino acids.

There has been considerable interest in the use of keto analogues of essential amino acids in liver and kidney disease (Walser, 1976; Maddrey, Weber & Walser, 1974; Maddrey et al., 1976; Richards, 1978). α-Keto acids can replace the corresponding amino acids in the diet of rats, with the exception of lysine and threonine (Richards, 1972). In theory, α-keto acids could offset both hyperammonaemia and protein deficiency in chronic liver disease with portal-systemic encephalopathy

by combining with amino group donors to yield essential amino acids. Amination of these acids has been observed in tissues other than liver, such as muscle, and this suggests that normal liver function may not be a prerequisite for the utilization of these compounds for protein synthesis. Studies on patients with chronic portal-systemic encephalopathy have supported this suggestion (Maddrey et al., 1976). In these patients, given keto analogues of five essential amino acids either parenterally or orally, the principal source of the nitrogen for conversion of the keto acids appeared to be glutamine. Plasma glycine and tyrosine concentrations returned toward normal during administration; no change was observed in arterial blood ammonia but there was a pronounced decrease in glutamine. No toxicity was found and clinical improvement occurred in 8 of 11 patients. An additional advantage of administering keto analogues of essential amino acids is that the branched chain keto acids may themselves promote protein synthesis, particularly in muscle (Sapir & Walser, 1977). The usefulness of the essential amino acid analogues in the treatment of hepatic failure has yet to be established. However, in view of the present results and many earlier experiments showing the detrimental effects of administering protein during hepatic failure, the possibilities of providing a nitrogen-free source of the essential amino acids should possibly receive more attention.

RESULTS SECTION III

BLOOD-BRAIN BARRIER: TRYPTOPHAN TRANSPORT DURING HEPATIC ENCEPHALOPATHY

The fate of tryptophan in the brain depends both on its concentration within specific regions of specific neurons where it is metabolized (mostly to protein and to a much lesser extent to serotonin), and on the true fluxes of its transport into brain, into specific neurons and finally into subcellular regions. Although other processes such as metabolism are undoubtedly important in determining the concentration and distribution of tryptophan in the brain, there is much evidence that transport is one of the main determinants. Since serotonin- if not protein synthesis appears to be limited by tryptophan availability (Carlsson, 1974; Friedman et al., 1972; Barondes, 1974), rates of transport may be important in regulating these processes (Lajtha, 1974; Pardridge, 1977). Of the various transport steps, entry from the plasma is believed to be rate-limiting since the predicted maximal velocity of tryptophan entry into brain cells is much higher than the rate of influx through the blood-brain barrier (Bauman, Bourgoin, Benda, Glowinski & Hamon, 1974; Parfitt & Graham-Smith, 1974) and extracellular fluid concentrations of all amino acids are much lower than cell concentrations (Bito, Davson, Levin, Murray & Snider, 1966; McGale, Pye, Stonier, Hutchinson & Aber, 1977). In addition, indirect evidence is provided by experiments showing that changes in the factors known to affect blood-brain barrier transport of tryptophan (such as competition by plasma amino acids and the plasma concentration of unbound tryptophan), result in predictable changes in serotonin concentrations and turnover in whole

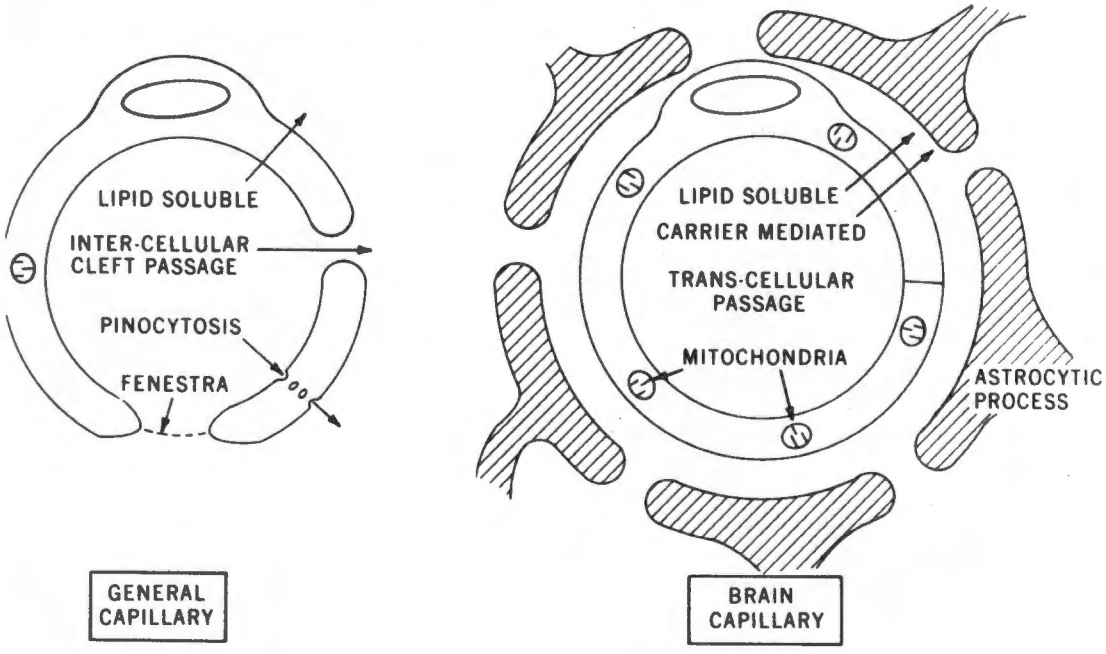
brain, as well as in regions with large numbers of serotoninergic neurons (Colmenares et al., 1975; Fernstrom & Wurtman, 1971a,b, 1972; Gessa & Tagliamonte, 1974; Tagliamonte et al., 1973b; Curzon & Knott, 1974a,b; Curzon, Joseph & Knott, 1972). Therefore the study of blood-brain barrier transport is vitally important for a greater understanding of changes in brain tryptophan and serotonin metabolism.

The site of the blood-brain barrier is believed to be the tight junctions between endothelial cells of the brain capillaries (Oldendorf, 1975; Pardridge, 1977) (Figure 18). These cells have no open clefts between them as do capillaries elsewhere in the body. There is also a marked absence of pinocytotic vesicles. Therefore, since substances must pass through two capillary cell walls to reach brain extracellular fluid, passage through the blood-brain barrier depends on either lipid solubility or mediation by carriers present in the capillary cell membranes. Most substrates pass by means of facilitated diffusion, i.e. the process is equilibrative (not concentrative), Na^+- and energy-independent, and stereospecific. Depending on the kinetic constants characterizing each system, different substrates transported by the same system may compete with each other for entry. Influx of substrates is therefore affected by the concentration of the substrate as well as the concentrations of the competitive inhibitors and the kinetic constants characterizing the system. When penetration is high, influx into the brain is also affected by cerebral blood flow, since at higher blood flows extraction of the substrate will be less complete.

In vivo studies of amino acid transport showed that most amino acids could be assigned to one of three carriers, one for the acidic amino acids glutamate and aspartate, one for the basic amino acids arginine,

FIGURE 18

DIAGRAM OF MAJOR DIFFERENCES BETWEEN A
GENERAL (NONNEURONAL) CAPILLARY AND A BRAIN CAPILLARY



(adapted from Oldendorf, 1975)

In the general capillary, small molecules can equilibrate between the moving extracellular fluid (plasma) and the stationary extracellular fluid outside by diffusion through the intercellular cleft. Pinocytosis is a relatively inefficient mechanism of permeability but probably is independent of molecular size and can pass even very large macromolecules. In the brain capillary, these nonspecific routes of exchange are missing; the intercellular clefts are sealed shut by tight junctions, there is markedly reduced pinocytosis and no fenestrae. Transcapillary exchange must accordingly take place through the cells of the capillary wall, the major barrier of which is the plasma membrane (inner and outer) of the capillary endothelial cells. This transcapillary exchange is therefore dependent on lipid solubility of the substrate and carrier mediated transport.

lysine and ornithine, and one for the neutral amino acids (Oldendorf, 1971b; Oldendorf & Szabo, 1976; Richter & Wainer, 1971). The essential amino acids were taken up to a much larger extent than the nonessential amino acids. Tryptophan transport is mediated by the neutral system which also carries tyrosine, phenylalanine, valine, leucine, isoleucine, methionine and threonine at similar influx rates.

Findings described in the previous section of the thesis, which indicated that plasma competition effects could not account for the brain amino acid changes observed during hepatic failure, prompted an investigation of possible alterations in the transport mechanism for tryptophan. In these studies the term transport is defined as the movement of an amino acid from blood to brain without suggesting any net increase in the quantity of amino acid in the brain and without consideration of the numerous compartments and membrane barriers that actually comprise the system.

A. METHODS

INTRODUCTION

Methods used to study blood-brain barrier transport involve either steady-state or single injection techniques. With the steady-state method, a steady level of radiolabeled test-substance is maintained in the circulation and the radioactivity in brain relative to that in plasma is measured at various times (Daniel, Donaldson & Pratt, 1975a). The single-injection technique involves the injection of test substance together with a reference which is either completely nondiffusible and indicates dilution of the injectate in blood (Crone, 1963, 1965; Yudilevitch & De Rose, 1971), or highly permeable in which case it is measured in brain tissue and indicates the amount of injectate reaching the portion of brain tested (Oldendorf, 1971a,b). The results obtained with the various methods agree fairly well with each other (Bãnos, Daniel, Moorhouse & Pratt, 1975; Pardridge & Oldendorf, 1975a). The method first described by Oldendorf was used for the present experiments because it was the simplest and could be used in rats. In this method the ^{14}C-amino acid is injected together with the reference ^{3}H-water into the common carotid of the rat (Figure 19). The injected bolus is assumed to remain unmixed with blood constituents during its passage through the brain. A known amount of ^{3}H-water and an unknown fraction of ^{14}C-amino acid enter the brain. The solution is washed out of the brain by the circulation before the rat is decapitated 15 seconds after injection. A sample of brain tissue and of injectate are counted and the brain uptake index (BUI) calculated according to:

FIGURE 19

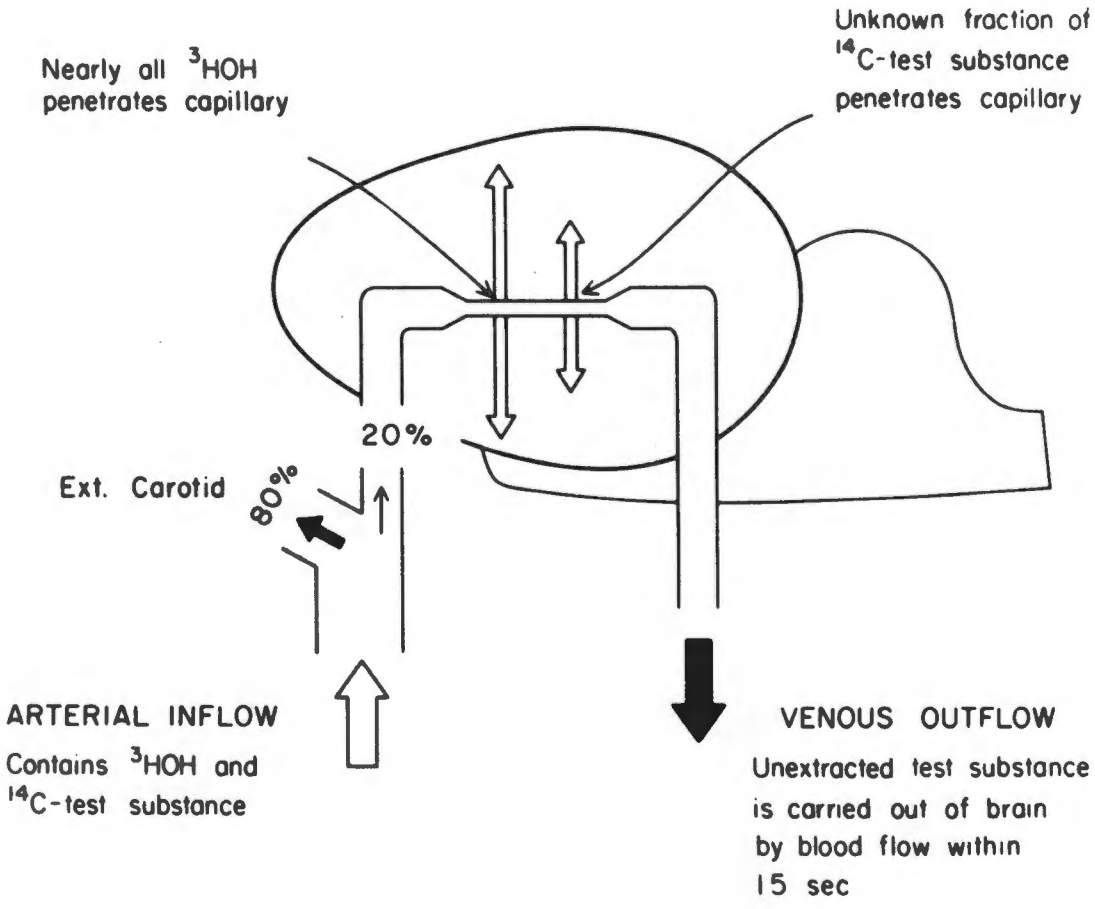


DIAGRAM OF METHOD USED IN DETERMINING BRAIN INFLUX OF A ^{14}C-AMINO ACID FROM A SINGLE CAPILLARY PASS

The mixture of a ^{14}C-labeled test substance and ^{3}H-water is injected rapidly into the rat common carotid artery and the rat is decapitated 15 seconds later. The test substance (^{14}C) remaining in brain is related to the reference (^{3}H) remaining. The mixture is washed out of the brain before decapitation. From Oldendorf (1971b).

$$BUI = E \times 100$$

$$\text{where E (fractional extraction)} = \frac{^{14}C/^{3}H \text{ brain tissue}}{^{14}C/^{3}H \text{ injectate}}$$

Several assumptions have to be made when using this procedure as originally described:

a) The rate of efflux of the test substance and the reference from the brain tissue must be the same during the time of the experiment.

b) The injectate must be completely removed from the brain capillaries and not recirculated during the time before decapitation. Oldendorf found a background BUI of 1-2% after 15 seconds (Oldendorf & Szabo, 1976) due almost entirely to incomplete brain-blood compartment washout with only a minor contribution from systemically recirculating radioisotope. Therefore when testing substrates with high BUI's such as the neutral amino acids, this background can be ignored. In order to test the above two assumptions under the conditions used, tryptophan transport was measured at 5, 10 and 15 seconds. The BUI's obtained were 19.0 ± 0.94, 17.9 ± 2.9, 15.7 ± 3.9 (means ± S.E.M. of three determinations) respectively. The higher values at shorter times suggest that brain washout was less complete; however the differences between the results were not significant.

c) There must be no variation in cerebral blood flow. This is necessary since the extraction is affected by blood flow according to the equation (Kety, 1951);

$$E = 1 - e^{-PS/F}$$

where PS = capillary permeability coefficient x surface area

F = cerebral blood flow

Blood flow in turn is affected by physiological parameters such as body temperature and blood gases (Hägerdal, Harp & Siesjö, 1975; Borgström, Johannsson & Siesjö, 1975). Therefore these parameters should remain unchanged during the experiment. In the presence study halothane anaesthesia was used instead of pentobarbital (as originally described by Oldendorf) which causes a profound reduction in cerebral blood flow, body temperature as well as increased circulating CO_2 and decreased O_2 (Nilsson & Siesjö, 1975). With halothane anaesthesia respiratory depression was minimal and body temperature maintained above 37°C for the duration of the experiment.

In addition, in order to compare different experimental groups, it is necessary that blood flow is constant in all conditions examined. Cerebral blood flow has been measured in rats with portacaval shunts and found to be indistinguishable from controls (Gjedde, Lockwood, Duffy & Plum, 1978; Eklöf, Holmin, Jóhannsson & Siesjö, 1974). The situation in acute hepatic failure is not clear. There have been no reports of measurements of blood flow in rats with acute hepatic failure; however in patients a slight increase was observed (Maiolo et al., 1971) and in goats with CCl_4-induced acute hepatic necrosis there was a decrease (Stanley, 1978). This is discussed further in the Results section.

d) The extraction of ^{3}H-water must be almost complete. This was subsequently found not to be the case. Various values for the fractional extraction have been reported, ranging from 0.5 to 0.9 at various blood flows and in different species (Brender, Andersen & Rafaelsen, 1975; Oldendorf & Braun, 1976; Raichle, Eichling & Grubb, 1974). For the present study the extraction of ^{3}H-water was separately determined relative to ^{14}C-n-butanol, which is almost completely equilibrated on passage through capillaries (Crone, 1965; Raichle, Eichling, Straatman, Welch, Larson & Ter-Pogossian, 1976), under the same conditions as used for studying tryptophan transport. The mean extraction of 18 determinations was found to be 0.545 ± 0.053 (S.E.M.). Therefore the BUI was calculated as

$$BUI = E \times 54.5$$

The use of a partially diffusable reference such as water has the advantage that its extraction will be affected by unknown alterations of blood flow to a similar extent as the test substance. Thus if a substance has an extraction of 0.2 at a flow of 1.5 $ml.min^{-1}.g^{-1}$ and changes of flow to 1.2 or 1.8 $ml.min.^{-1}g^{-1}$ were to occur, it can be calculated that the extraction would change to 0.21 and 0.19 respectively. If, on the other hand, a perfectly diffusible reference were used, such as n-butanol, extractions of 0.24, 0.20 and 0.17 would be found at blood flows of 1.2, 1.5 and 1.8 $ml.min.^{-1}g^{-1}$, respectively.

PROCEDURE

Tryptophan transport was studied in normal rats, sham-operated controls, after portacaval shunt alone, after hepatic devascularization (acute hepatic failure), and four weeks after portacaval shunt (chronic hepatic failure). The experimental procedures for these are fully described in the Experimental Section. In the acute hepatic failure condition, transport was measured three hours after ligation of the hepatic artery at which time the brain metabolites showed all the abnormalities (including increased tryptophan concentrations) found in rats sampled in the terminal stages preceding death. Results were compared to those obtained from normal rats after establishing that the findings in sham-operated controls were not different from normal.

The rat was anaesthetised with 1.5% halothane in oxygen, and the left carotid artery exposed. After positioning the rat on a guillotine, the artery was lifted slightly with a small paperclip to enable injection without occluding the flow of blood. Using a slightly bent needle (26 wire gauge, outer diameter 0.38 mm), 0.2 ml of radioactive solution was injected rapidly to minimise mixing with plasma. The artery cleared momentarily as the solution entered. Normal blood flow continued past the needle which was left in place until decapitation 15 seconds after injection. The whole procedure from induction of anaesthesia to decapitation took 8-10 minutes. The brain was rapidly removed and two samples of about 100 mg each of the left cortex removed. The remaining brain tissue was stored in liquid nitrogen at -196°C until extraction and assay of tryptophan. The cortex samples were placed in scintillation counting vials containing 1 ml of tissue solubilizer, Soluene-350. After standing overnight at room temperature

to allow complete solubilization, 10 ml of liquid scintillation cocktail, Dimilume-30, was added to each vial and the ^{14}C dpm/^{3}H dpm ratio determined on a Packard Tri-Carb scintillation counter, using a program which calculated disintegrations per minute from counts per minute, from a previously calculated quench curve. A sample of the injected solution was treated in the same way and counted to determine the original $^{14}C/^{3}H$ ratio.

The Soluene-350/Dimilume-30 system was preferred to others since it produced less colour, and chemiluminescence was more rapidly inhibited due to the low pH of Dimilume (which neutralized the highly basic tissue solubilizer) and the presence of radical scavengers. Thus vials could be counted within a few hours of addition of scintillant.

The injected solution contained 1.25 µCi of [^{14}C]-tryptophan and 5 µCi [^{3}H]-water per ml, in a diluent consisting of Ringers solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 2 mM) containing 4 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid) as buffer, at pH 7.4. The solution was injected at room temperature. To examine the effects of increasing tryptophan concentration on transport, unlabeled tryptophan was added to obtain final concentrations ranging from 24 µM to 1980 µM tryptophan. In studies assessing the influence of competing amino acids, solutions were made containing 24 µM [^{14}C]-tryptophan and unlabeled leucine 130 µM, isoleucine 66 µM, valine 190 µM, methionine 47 µM, phenylalanine 67 µM, and tyrosine 63 µM.

In the preliminary experiments measuring the extraction of water relative to n-butanol, the solution contained 1.25 µCi of [^{14}C]-n-butanol and 5 µCi [^{3}H]-water in the HEPES-Ringers buffer.

CALCULATIONS

The brain uptake index (BUI) was calculated as follows:

$$BUI = \frac{^{14}C/^{3}H \text{ brain tissue}}{^{14}C/^{3}H \text{ injectate}} \times 54.5$$

The rate of tryptophan transport from the solution was calculated according to the formula:

$$\text{Influx } (\text{nmol.min}^{-1}.\text{g}^{-1}) = \frac{BUI}{100} \times \text{tryptophan concentration } (\mu M) \times \text{cerebral blood flow } (\text{ml.min}^{-1}.\text{g}^{-1})$$

The value for the cerebral blood flow used was 1.0 $\text{ml.min}^{-1}.\text{g}^{-1}$ since this was the approximate mean found in normal rats and after porta-caval shunt (Eklöf et al., 1974; Gjedde et al., 1978; Norberg & Siesjö, 1974).

Mediated transport is similar in many respects to an enzymatic reaction. Thus, analysis of these transport data can be simplified by assuming that the substance outside, the carrier, and the solute inside the brain, are analogous to the substrate, enzyme, and product, of an enzymatic reaction, respectively. When the rate of influx has been determined over a range of concentrations, the results can be analysed using enzyme kinetic techniques. The K_m and V_{max} for transport are calculated by fitting the data to the Michaelis-Menton equation:

$$v = \frac{V_{max}S}{S + K_m}$$

where v = rate of influx

S = substrate concentration

V_{max} = maximal influx rate

K_m = Michaelis constant

The K_m for an enzymatic reaction provides a means by which the degree of saturation of the catalytic sites on the enzyme can be estimated.

In the presence of a competitive inhibitor the K_m is apparently increased by the factor $(1 + \frac{I}{K_I})$ where I = inhibitor and K_I is the inhibition constant, i.e.:

$$\text{influx} = \frac{V_{max}S}{S + K_m\ (1 + \frac{I}{K_I})}$$

and when several competitors are present:

$$\text{influx} = \frac{V_{max}S}{S + K_m\ (1 + \Sigma\ \frac{I}{K_I}\ )}$$

where $\Sigma\ \frac{I}{K_I}$ is the sum of the ratios of each competitor to its K_I. For the amino acid transport system it has been shown that the K_Is of competing amino acids are equivalent to their K_ms of transport, i.e. they are true competitive inhibitors (Christensen, 1969). Therefore published values for the K_ms can be used in the equation.

B. RESULTS

KINETICS OF TRYPTOPHAN INFLUX AND ALTERATIONS DURING ACUTE HEPATIC FAILURE

After hepatic devascularization, the brain uptake index (BUI) and calculated influx of tryptophan were about twice as high as in control rats at all concentrations of tryptophan tested (Table 22). As the concentration of unlabeled tryptophan was increased, the BUI decreased (Figure 20) to a minimum. This indicated that the transport system was saturable, i.e. the increasing concentration of unlabeled tryptophan was competing with the ^{14}C-tryptophan (the concentration of which remained constant) and inhibiting its transport. The effect on influx is shown in Figure 21. At lower concentrations a hyperbolic curve characteristic of saturation kinetics was observed but saturation was not reached even with concentrations many times those found normally. At the higher concentrations influx appeared to be directly proportional to concentration. A double reciprocal plot (Figure 22) was the next step in trying to explain these findings. In both normal and acute hepatic failure conditions the plot curved towards the origin at higher concentrations. This nonlinearity suggested that more than one system was operating (Denizeau, Wyse & Sourkes, 1976), with the influx observed being the sum of the various components at each concentration. The simplest possibilities to explain the data were the following:

1) two saturable facilitated diffusion transport systems (one with a low V_{max} and low K_m and another with a high V_{max} and high K_m).

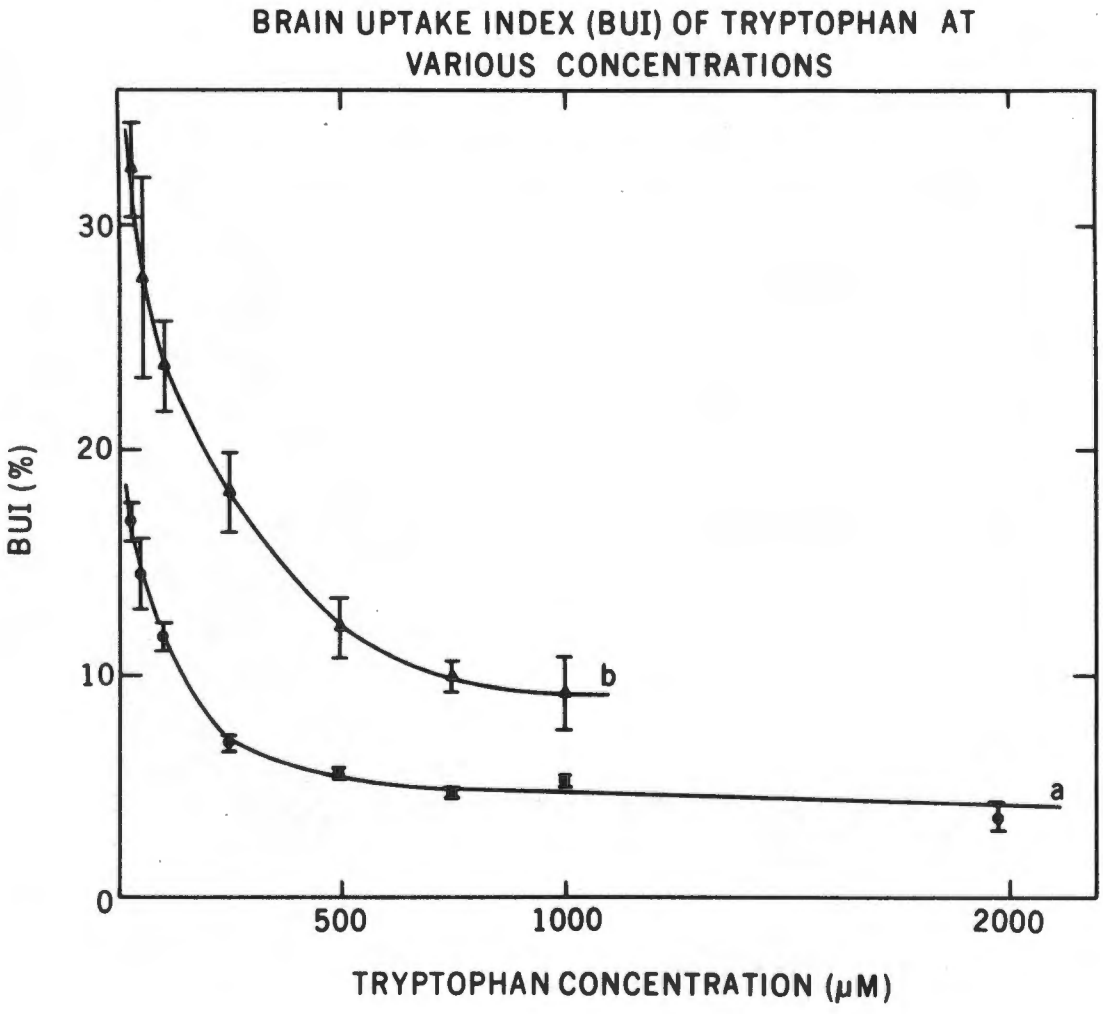
TABLE 22

TRYPTOPHAN INFLUX IN NORMAL RATS AND DURING ACUTE HEPATIC FAILURE

For experimental details see the Procedure section. The brain uptake index (BUI) was calculated as $\frac{^{14}C/^{3}H \text{ (tissue)}}{^{14}C/^{3}H \text{ (injectate)}} \times 54.5$ and the influx as $\frac{BUI}{100} \times$ (concentration) $\times$ (blood flow). The value for cerebral blood flow used was 1.0 ml. $min^{-1}.g^{-1}$. The results are expressed as the mean ± S.E.M. with the number of rats in parenthesis. Results significantly different from normal values are indicated by *p<0.01, **p<0.001.

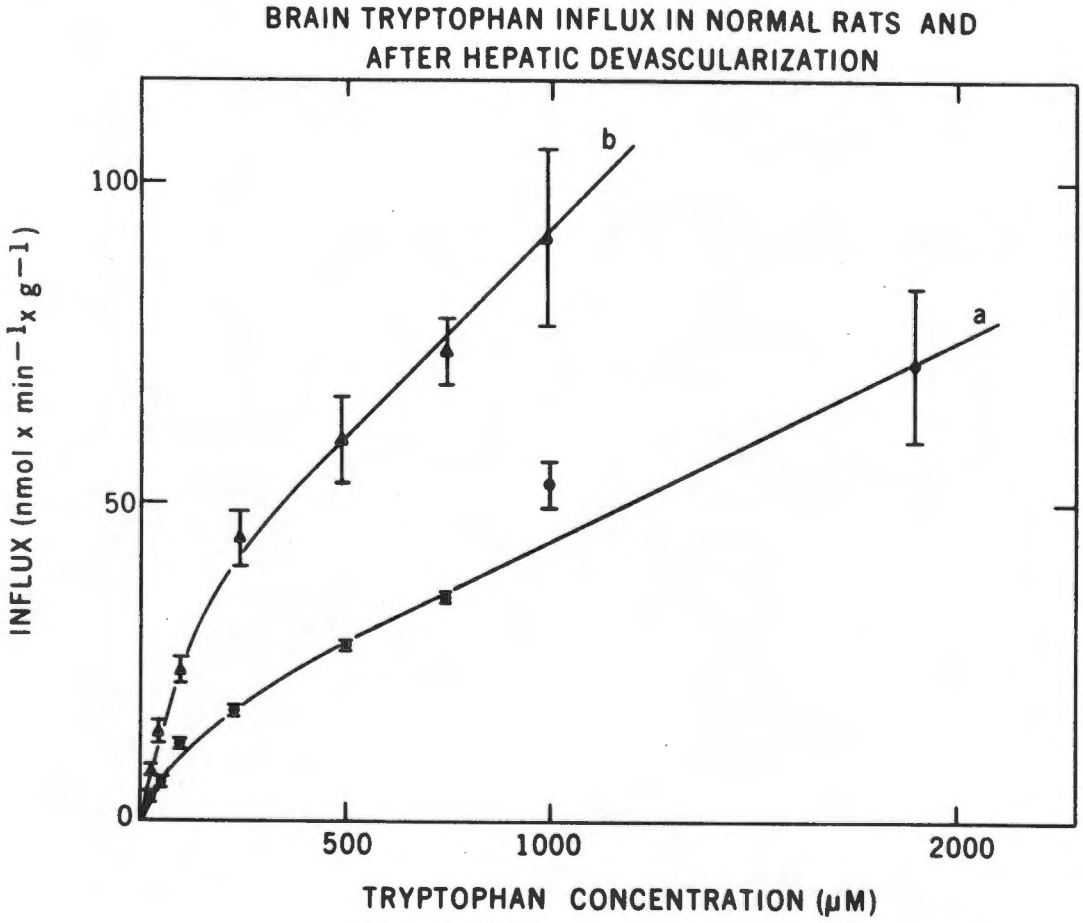
Tryptophan Concentration (μM)	Normal			Acute hepatic failure			% of normal
	BUI	Influx ($nmol.min^{-1}.g^{-1}$)		BUI	Influx ($nmol.min^{-1}.g^{-1}$)		
24	16.8 ± 0.9	4.02 ± 0.21	(16)	32.7 ± 2.1**	7.85 ± 0.50	(3)**	195
49	14.4 ± 1.6	7.07 ± 0.78	(6)	27.8 ± 4.5*	13.6 ± 2.2	(3)*	192
98	11.7 ± 0.6	11.5 ± 0.6	(7)	23.8 ± 1.9**	23.3 ± 1.9	(3)**	202
243	6.87 ± 0.39	16.7 ± 0.9	(12)	18.2 ± 1.7**	44.3 ± 4.1	(5)**	265
494	5.59 ± 0.26	27.6 ± 1.3	(7)	12.1 ± 1.4**	59.7 ± 6.9	(5)**	216
744	4.70 ± 0.13	35.0 ± 1.0	(7)	9.92 ± 0.65**	73.8 ± 4.8	(7)**	211
998	5.25 ± 0.39	52.4 ± 3.8	(6)	9.13 ± 1.70**	91.1 ± 17.0	(5)**	174
1980	3.64 ± 0.65	72.0 ± 12.8	(5)	-	-		-

FIGURE 20



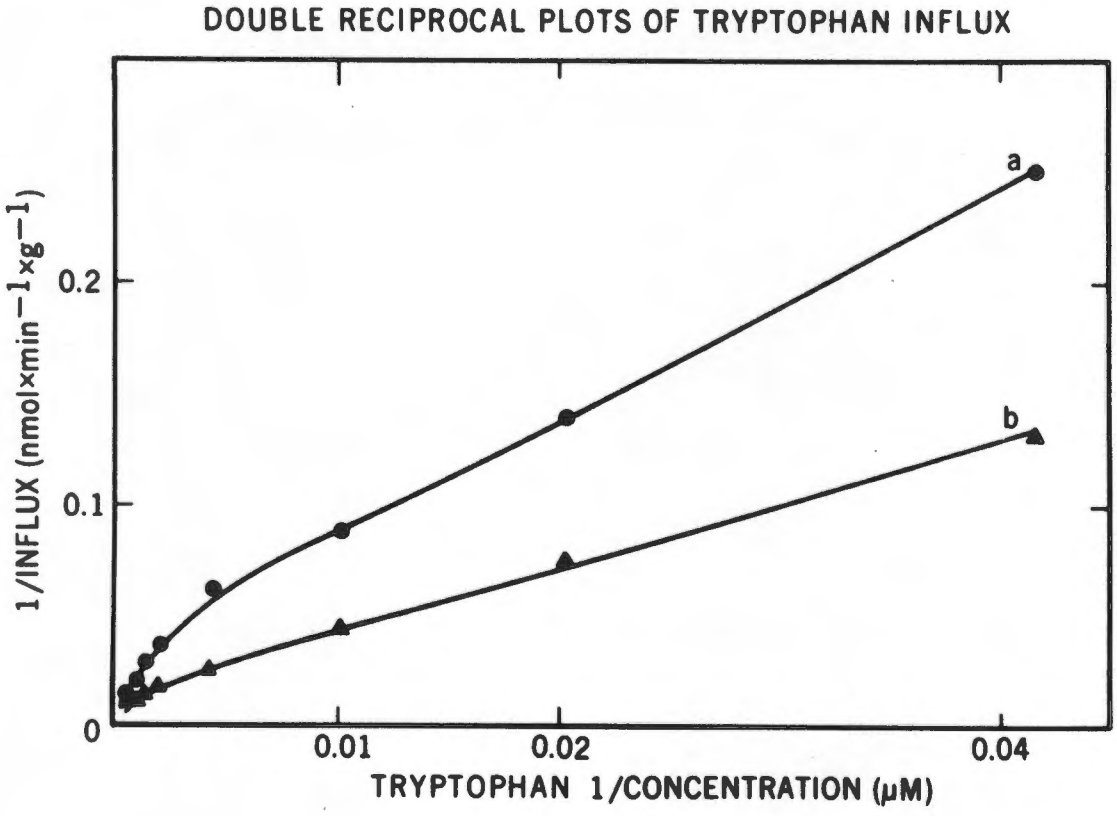
For experimental details and calculation of the BUI see Table 22. The points are the data given in Table 22 and represent the means of at least five determinations (line a, normal rats) or three determinations (line b, acute hepatic failure). The S.E.M. is indicated by the vertical bars.

FIGURE 21



Influx was calculated as described in Table 22. The points represent the means of at least five determinations in normal rats (line a) or three determinations in rats with acute hepatic failure (line b). The vertical bars represent the S.E.M.

FIGURE 22



The lines were plotted using the reciprocals of the data in Table 22. Line a indicates results from normal rats, line b from those with acute hepatic failure.

2) one saturable facilitated system (with low V_{max} and low K_m) and free (non-facilitated) diffusion.

An approximation of the two components can be obtained by drawing tangents to the curves at low and high concentrations. However this method involves a large error which can be removed by subtracting each component from the other iteratively until the contribution made by component 1 to component 2 is negligible and vice versa (Spears, Sneyd & Loten, 1971). By following this procedure the data of Table 22 were resolved into the various components shown on Tables 23 and 24 together with the kinetic constants describing the systems. The close correlation between the sum of the calculated components and the actual influx obtained indicated the completeness of the resolution.

The high capacity component in each case was described by a proportionality constant K (i.e., assuming a linear relationship) since no definite distinction could be made between this possibility and a system with very high V_{max} and K_m. Measurements at even higher concentrations would be needed to determine whether this system is eventually saturable or represents free diffusion.

During acute hepatic failure the affinity constant (K_m) of the low capacity component was similar to that in normal rats, while the transport capacity (V_{max}) was considerably greater. The constant of transport by the high capacity component was increased more than two-fold. (If this component were to be described by a K_m and V_{max}, the K_m would be about 80% of normal, i.e. the affinity would be increased, and the V_{max} more than doubled.) The contributions made by the two components at various concentrations are shown in Figure 23. At plasma tryptophan concentrations found in vivo, i.e. below 200 μM,

TABLE 23

TWO COMPONENTS OF TRYPTOPHAN TRANSPORT IN NORMAL RATS

The data of Table 22 were resolved into two components by an iterative subtraction procedure (Spears et al., 1971). The kinetic constants were determined from the slopes and intercepts of the two lines thus obtained.

	Calculated Components of Influx ($nmol.min^{-1}.g^{-1}$)			
Tryptophan Concentration (μM)	Low capacity component	High capacity component	Sum of two components	Observed Influx ($nmol.min^{-1}.g^{-1}$)
24	3.45	0.564	4.01	4.02
49	5.92	1.16	7.08	7.07
98	9.21	2.34	11.5	11.5
243	13.4	5.92	19.3	16.7
494	16.1	12.5	28.6	27.6
744	17.1	17.9	35.0	35.0
998	17.7	34.6	52.3	52.4
1980	18.7	53.3	72.0	72.0

Kinetic constants

K_m: 113 μM (low capacity component)

V_{max}: 19.5 $nmol.min^{-1}.g^{-1}$ (low capacity component)

K: 0.026 $ml.min^{-1}.g^{-1}$ (high capacity component)

TABLE 24

TWO COMPONENTS OF TRYPTOPHAN TRANSPORT IN RATS WITH ACUTE HEPATIC FAILURE

For details, see Table 23.

Tryptophan Concentration (μM)	Calculated Components of Influx ($nmol.min^{-1}.g^{-1}$)			Observed Influx ($nmol.min^{-1}.g^{-1}$)
	Low capacity component	High capacity component	Sum of two components	
24	6.16	1.69	7.85	7.85
48	10.2	3.37	13.6	13.6
98	16.6	6.75	23.4	23.3
243	23.6	16.7	40.3	44.3
494	27.7	32.0	59.7	59.7
744	29.5	44.2	73.7	73.8
998	30.4	60.7	91.1	91.1

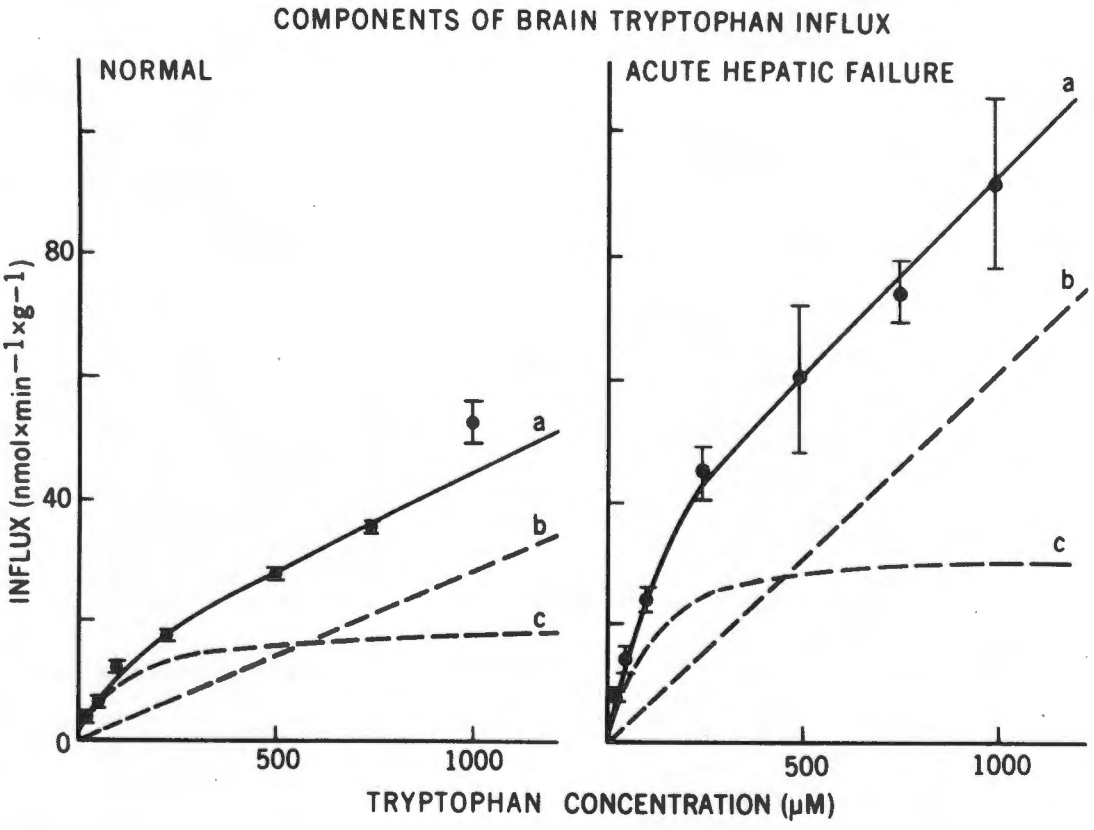
Kinetic constants

K_m: 108 μM (low capacity component)

V_{max}: 33.8 $nmol.min^{-1}.g^{-1}$ (low capacity component)

K: 0.067 $ml.min^{-1}.g^{-1}$ (high capacity component)

FIGURE 23



The data of Table 22 were analyzed by an iterative procedure to separate the influx into two components as described in the text. Line a is drawn through the experimentally obtained points. Each point is the mean of at least five (normal) or three (acute hepatic failure) determinations with the S.E.M. indicated by the vertical bars. Lines b and c represent the high and low capacity components, respectively.

the major fraction of tryptophan entry into the brain would be mediated by the low capacity saturable component of transport in both conditions.

THE EFFECT OF COMPETING AMINO ACIDS

In the above experiments tryptophan influx was measured in the absence of any amino acids that would normally compete with it for entry in vivo. This was necessary in order to obtain the kinetic constants for tryptophan transport. To obtain estimates of tryptophan influx in physiological conditions, predictions were made of the actual influx occurring in normal rats and during acute hepatic failure, at various hypothetical tryptophan concentrations, using the kinetic constants for tryptophan influx obtained for the two components and the competing amino acid concentrations found in these conditions (Table 26). The high capacity component would not be affected at the concentrations of substrates involved even if it were eventually saturable, due to its very low affinity and high capacity. Thus total influx was calculated as

$$\text{influx} = \frac{V_{max}S}{S + K_m\left(1 + \Sigma \frac{I}{K_I}\right)} + KS$$

These calculations showed that in normal rats about 40% of the influx is mediated by the high capacity component while this is increased to about 74% during acute hepatic failure. This means that any further increase in plasma competitor concentration would have a minor effect in inhibiting tryptophan transport since entry could be reduced by only 26% at the most. The individual effects of the altered kinetics

TABLE 25

PREDICTED TRYPTOPHAN INFLUX IN THE PRESENCE OF CONCENTRATIONS OF COMPETING AMINO ACIDS FOUND IN NORMAL RATS AND DURING ACUTE HEPATIC FAILURE

Influx by the low capacity component was calculated using the equation: $\text{influx} = \frac{V_{max} S}{S + K_m (1 + \Sigma \frac{I}{K_m})}$ where $\Sigma \frac{I}{K_m}$ is the sum of the ratios of the concentration of each competing amino acid to its K_m. The kinetic constants for tryptophan are shown in Tables 23 and 24. The concentrations of the competing amino acids used in the calculation are given in Table 28 while K_ms were taken from Pardridge & Oldendorf (1975a): tyrosine 160 μM, phenylalanine 120 μM, methionine 190 μM, leucine 150 μM, isoleucine 330 μM, valine 630 μM, threonine 730 μM. (Since the K_m for tryptophan was essentially unchanged during hepatic failure, it was assumed that the K_ms for the other amino acids were similarly unchanged.)

Tryptophan Concentration (μM)	Influx predicted in normal rats ($nmol.min^{-1}.g^{-1}$)				Influx predicted during acute hepatic failure ($nmol.min^{-1}.g^{-1}$)				
	Low capacity system	High capacity system	Total	% by high system	Low capacity system	High capacity system	Total	% by high system	% of normal (total)
25	1.1	0.7	1.8	39	0.56	1.7	2.3	74	128
50	2.1	1.3	3.4	38	1.1	3.4	4.5	76	132
100	3.8	2.6	6.4	41	2.1	6.7	8.8	76	138
200	6.3	5.2	11.5	45	4.0	13.4	17.4	77	151

and high plasma concentrations of competing amino acids in acute hepatic failure were examined by including these factors singly in the calculation (Table 26). Total influx at 50 μM tryptophan is reduced from 3.4 to 1.9 nmol.min^{-1}.g^{-1} by the high plasma competitors (when the kinetic constants are held normal). The altered kinetic constants on the other hand, increase total influx from 3.4 to 7.2 nmol.min.$^{-1}$.g^{-1}. The combined effects result in a net increase of influx from 3.4 to 4.6 nmol.min.$^{-1}$.g^{-1}.

In order to test these predictions in vivo, competing amino acids were added to the injected solution to obtain concentrations approximating those normally found in rat plasma. This resulted in a reduction of tryptophan influx from 4.02 to 1.69 nmol.min.$^{-1}$.g^{-1} in normal rats and from 7.85 to 3.31 in rats after hepatic devascularization (Table 27). The values obtained corresponded well with those predicted from calculations of the influx where the inhibitory effect of the competitors on transport by the low capacity component was taken into account, thus confirming the validity of the method of calculation and the kinetic constants obtained.

CORRELATION WITH BRAIN TRYPTOPHAN CONTENT

Tryptophan influx was found to be increased when measured after portacaval shunt alone although not as much as after both portacaval shunt and hepatic artery ligation (Table 29). A significant correlation was found between brain tryptophan content and influx from 24 μM tryptophan solution in the three groups of rats: normal, portacaval shunt alone and acute hepatic failure (Table 29, Figure 24), providing

TABLE 26

INDIVIDUAL EFFECTS OF ALTERED TRANSPORT KINETICS AND ALTERED COMPETITOR CONCENTRATIONS ON TRYPTOPHAN TRANSPORT

Influx was calculated (equation in Table 25) using combinations of kinetic constants and plasma competing amino acid concentrations found in normal rats (Table 23, Table 28) and during acute hepatic failure (Table 24, Table 28).

	Predicted Influx ($nmol.min^{-1}.g^{-1}$) at 50 μM Tryptophan		
	Low component	High component	Total
1) Normal kinetic constants + normal competitors	2.1	1.3	3.4
2) Normal kinetic constants + high competitors	0.6	1.3	1.9
3) High kinetic constants + normal competitors	3.8	3.4	7.2
4) High kinetic constants + high competitors	1.1	3.4	4.5

TABLE 27

EFFECT OF COMPETING AMINO ACIDS ON TRYPTOPHAN INFLUX

Influx was determined as described in the Procedure section. The solution used to measure influx in the presence of competitors contained tyrosine 63 μM, phenylalanine 67 μM, methionine 47 μM, leucine 130 μM, isoleucine 66 μM, valine 190 μM, in addition to tryptophan 22 μM. The results are expressed as the mean ± S.E.M. with the number of determinations in parenthesis.

	Influx ($nmol.min^{-1}.g^{-1}$)	
	Normal	Acute hepatic failure
Tryptophan (22 μM)	4.02 ± 0.26 (16)	7.85 ± 0.52 (3)
Tryptophan (22 μM) + competitors	1.69 ± 0.07 (5)	3.31 ± 0.07 (3)
Predicted influx with competitors*	1.58	3.30

* calculated using the equation: $\text{influx} = \frac{V_{max}S}{S + K_m(1 + \Sigma\frac{I}{K_m})} + KS$

where the constants for tryptophan were those in Tables 23 and 24, the K_ms for the amino acids those given in Table 25, and the concentrations of the amino acids as given above.

TABLE 28

CONCENTRATIONS OF AMINO ACIDS USED IN CALCULATIONS FOR TABLES 25, 26, and 27.

Concentrations are given in μM.

Amino Acid	Added to injectate[1]	Normal[2]	Acute hepatic failure[3]
Tyrosine	63	60	410
Phenylalanine	67	70	420
Methionine	47	40	280
Valine	190	170	310
Leucine	130	130	250
Isoleucine	66	60	150
Threonine	-	150	550

[1] Used to study the effects of competitors in influx (see Table 27).

[2] Found in plasma of control rats of hepatic failure studies described in Results Section II.

[3] Found in plasma during acute hepatic failure studies described in Results Section II.

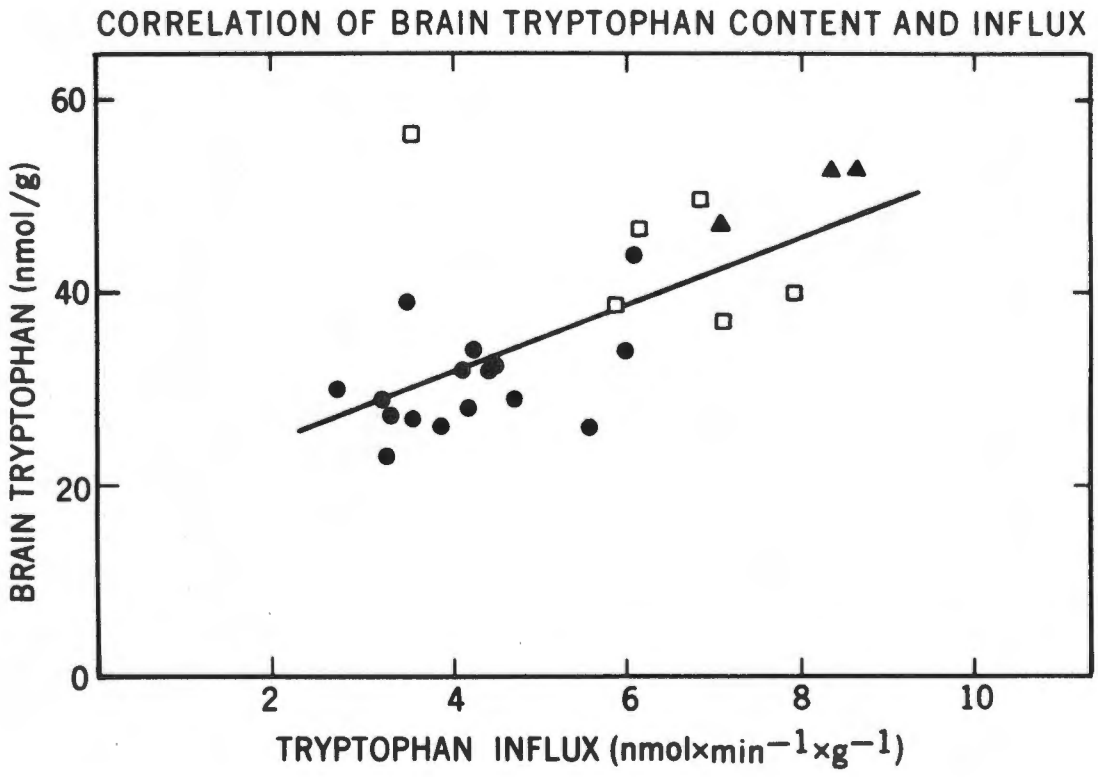
TABLE 29

CORRELATION BETWEEN BRAIN TRYPTOPHAN CONTENT AND INFLUX OF TRYPTOPHAN AFTER PORTACAVAL SHUNT ALONE AND AFTER HEPATIC DEVASCULARIZATION

Rats were injected 65 hours after portacaval shunting or 3 hours after hepatic artery ligation (acute hepatic failure group). The results are expressed as mean ± S.E.M. with the number of rats in each group in parenthesis. Results significantly different from normal values are indicated by *$p<0.01$, **$p<0.001$.

	Normal	Portacaval shunt alone	Acute hepatic failure
Brain tryptophan (nmol/g wet wt tissue)	30.2 ± 1.3 (16)	43.9 ± 3.9 (6)*	49.1 ± 1.9 (3)**
Percent of normal		145	162
Tryptophan influx ($nmol.min^{-1}.g^{-1}$ from 24 μM solution)	4.02 ± 0.26 (16)	5.90 ± 0.26 (6)*	7.85 ± 0.52 (3)**
Percent of normal		147	195
Correlation coefficient (for all rats)	$r = 0.64$ ($p<0.001$ that $r = 0$)		

FIGURE 24



Brain tryptophan content was compared to tryptophan influx in normal rats (●); after portacaval shunt alone (◘) and after hepatic devascularization (▲). The correlation coefficient r = 0.64 ($p<0.001$).

evidence of the importance of blood-brain barrier transport of tryptophan for total brain tryptophan content.

TRYPTOPHAN INFLUX IN CHRONIC HEPATIC FAILURE

When measured four weeks after portacaval shunt, tryptophan influx from a 22 μM solution showed a 32% increase from normal. The mean value obtained from 5 determinations was 5.3 ± 0.4 (S.E.M.) $nmol.min^{-1}.g^{-1}$ ($p < 0.05$, when compared to normal). This value is similar to that seen 65 hours after shunting (5.9 ± 0.3 $nmol.min^{-1}.g^{-1}$).

CEREBRAL BLOOD FLOW

Reports in the literature about changes in cerebral blood flow during hepatic encephalopathy have been conflicting. A decrease was found in patients with chronic hepatic failure (Maiolo, 1971) but not in rats with portacaval shunts (Gjedde et al., 1978; Eklöf et al., 1974). In patients with acute hepatic failure, there was a slight increase (Maiolo et al., 1971) but a decrease in goats in coma from CCl_4-poisoning (Stanley, 1978). The present studies were carried out three hours after hepatic artery ligation before the terminal stage preceding death. Since these rats were still conscious it was considered unlikely that blood flow would be altered. In order to account for the doubling of tryptophan transport observed, blood flow would have had to decrease from 1 to 0.44 $ml.min.^{-1}.g^{-1}$. In addition, as explained in the Methods section, any change in blood flow would also have affected the ^3H-water reference and a decrease would therefore have to be even greater in order to explain the results. These considerations, as well as the finding of increased tryptophan transport after

portacaval shunt alone, when there is no change in cerebral blood flow (Gjedde et al., 1978) suggested that alterations in cerebral blood flow could not be significantly involved in producing the changes in transport observed.

C. DISCUSSION

Many experiments have shown that alterations in plasma concentrations of the neutral amino acids tyrosine, phenylalanine, leucine, isoleucine and valine, relative to tryptophan, influence brain tryptophan content in normal rats (Fernstrom & Wurtman, 1972; Etienne et al., 1976; Pérez-Cruet et al., 1974). Thus the concept arose that competition influences tryptophan transport and thereby overall uptake. With this in mind, it was suggested that the decreased plasma branched chain amino acid concentrations found during chronic hepatic failure were responsible for causing increased brain tryptophan content (Munro et al., 1975; James et al., 1976; Soeters & Fischer, 1976). However, concentrations of phenylalanine and tyrosine, also competitors of tryptophan transport, were increased in this condition, casting some doubt on this hypothesis (Daniel, Love, Moorhouse & Pratt, 1975b; Lal et al., 1975). In fact when influx from an arbitrarily chosen tryptophan concentration of 50 μM is calculated using data from James et al. (1976) and kinetic constants found in the present studies in normal rats, influx is unchanged in the rats four weeks after portacaval shunt compared to the normal rats (both about 2.9 $nmol.min^{-1}.g^{-1}$). Similarly when using the plasma amino acid concentrations obtained four weeks after portacaval shunting, described in Results Section II, influx in shunted rats is not altered (3.3 $nmol.min^{-1}.g^{-1}$) from normal (3.4 $nmol.min^{-1}.g^{-1}$). In the acute hepatic failure series it is even more obvious that the increase in brain tryptophan content cannot be attributed to decreased competition for passage into brain since all the competitors are increased. The finding of altered brain tryptophan transport during

hepatic failure described in this section is therefore of great significance when considering the factors contributing to the increased brain tryptophan content, as well as abnormalities in other brain amino acid and hence neurotransmitter concentrations.

SELECTIVE CHANGES IN BARRIER TRANSPORT

Dual component transport systems have been described for several substrates in brain tissue, e.g. tryptophan uptake into synaptosomes (Baumann et al., 1974), glutamate and aspartate transport into synaptosomes of cerebral cortex (Logan & Snyder, 1972), glucose and choline transport across the blood-brain barrier (Pardridge & Oldendorf, 1975b; Cornford, Braun & Oldendorf, 1978). The finding that tryptophan transport across the blood-brain barrier contains several components is consistent with these reports. Over the range of concentrations tested, the data did not show saturation of the high capacity system, thus it was impossible to distinguish between the possibilities of non-facilitated diffusion or a facilitated transport carrier with a high V_{max} and a low affinity. Therefore, the increased transport observed during acute hepatic failure may have been due to either a general increase in permeability of the blood-brain barrier or more selective alterations in the transport carrier. Earlier studies showing different alterations in the different carrier systems for blood-brain barrier transport during hepatic failure indicate selective changes rather than a generalized breakdown. For example, transport of D-glucose was unaltered 1-4 weeks after portacaval shunt while that of the basic amino acid arginine and the monocarboxylic acids pyruvate and butyrate was decreased (Cremer et al., 1977; James, Escourrou & Fischer, 1978; Sarna, Bradbury,

Cremer, Lai & Teal, 1979). These substrates are transported by different carrier systems from the neutral amino acid system that transports tryptophan. On the other hand, barrier permeability to normally non-permeable substances such as [^{51}Cr]-EDTA was unchanged (Sarna, Bradbury & Cavanagh, 1977). If there was a generalized breakdown of the blood-brain barrier under these circumstances one would expect the transport of other small molecules to be increased.

When considering tryptophan transport on its own it may seem irrelevant whether the high capacity component is due to free diffusion or facilitated diffusion, since at physiological concentrations influx is proportional to concentration. However, one important difference between the two possibilities is that a facilitated mechanism is likely to be stereo- and group-specific while free diffusion has no such limitations except possibly those associated with physico-chemical properties such as lipid solubility. Therefore this distinction between free and facilitated diffusion is important in the physiological situation, although difficult to make in many cases (Denizeau et al., 1976).

CONSEQUENCES OF TWO TRANSPORT COMPONENTS

The presence of a high capacity component which permits entry of tryptophan in direct proportion to concentration may be especially significant for total influx rates, since the effect of this is to diminish the importance of competition among the neutral amino acids, which affects the low capacity component only. This is clear from Table 25 in which calculated rates of tryptophan entry by each component are shown at concentrations of tryptophan in the physiological range,

in the presence of competing amino acids. It can be seen that by taking the competing amino acids into account, the predicted contribution by the high capacity component in normal rats is about 40%. This supports the concept that competition may influence tryptophan transport, by affecting the low capacity component. However, the effect of the unsaturated component is to permit some transport to occur in direct proportion to the concentration of non-albumin bound tryptophan. Thus, regardless of the concentrations of other neutral amino acids, tryptophan entry could never be reduced by more than 60% of normal. Rats with hepatic devascularization (and probably to a lesser extent rats with portacaval shunt alone), are even less sensitive to alterations in the concentration of other neutral amino acids. In these rats, a remarkable 74% of tryptophan influx appears to be mediated by the high capacity component and therefore tryptophan entry into the brain could never be reduced by more than 26%. It is important to realize that the effect of increasing competition on total influx becomes increasingly less significant as the concentrations of competitors increase, due to the corresponding decrease in the contribution made by the saturable component to total influx. For example, in normal rats, increasing competition from zero to approximately normal, decreased influx by about 58% (Table 27) while increasing competition from normal to high levels was predicted to decrease influx by 44% (Table 26). Thus, in the situation of acute hepatic failure, the very high concentrations of plasma competitors, as well as the altered kinetic constants, result in a very low contribution by the saturable component to total influx, and therefore great resistance to the effects of further increases in competition.

From these estimates it seems that adding more competing amino acids to a circulation already flooded with amino acids, to further inhibit tryptophan entry and thus lower brain tryptophan content, would be of little benefit, even though a reduction of as little as 26% may be important. In chronic failure the situation is different since here the plasma amino acid concentrations are not far from normal. The kinetics of transport are likely to have altered less significantly than during acute hepatic failure, judging from the lesser increase in influx observed. Thus even if the abnormal plasma amino acid pattern is not responsible for the increased brain tryptophan content, some significant reduction in influx may be achieved by increasing circulating competitor concentrations. Beneficial results have been obtained from infusing amino acid mixtures high in concentrations of branched chain amino acids to dogs and rats with portacaval shunts (Fischer et al., 1975; Rosen et al., 1978). However it was not clearly established that the improvements observed were due specifically to the amino acids present in the intravenous solution administered (see Section II). It is possible that merely normalizing plasma amino acids may not be sufficient; higher competitor concentrations may be necessary to overcome the effect of the altered transport kinetics.

CORRELATION WITH BRAIN TRYPTOPHAN CONTENT

The significant positive correlation between changes in brain tryptophan content and influx provides evidence of the possible relationship between the two findings. As will be discussed further in the next section, tryptophan transport into the brain appears to be a major determinant of brain tryptophan content. However a cause and

effect relationship cannot be definitely assumed in the absence of more information; for example, decreased protein synthesis or increased catabolism could also lead to a build-up of brain tryptophan, although this would lead to similar increases in other amino acids, such as the branched chain amino acids, in the brain. These amino acids showed no or very minor increases during hepatic failure (see Results Section II).

CONCLUSIONS

The changes in the transport kinetics observed during acute hepatic failure should result in increased influx of tryptophan, and could explain, at least partially, the high brain tryptophan content found in these rats in the presence of high concentrations of plasma competing amino acids. The high competitor concentrations cause a reduction in tryptophan transport by the low capacity system. However, total tryptophan entry across the blood-brain barrier is increased because of the altered kinetics of transport, i.e. the increased transport capacity (V_{max}) of the low capacity component reduces the effect of competition, and transport by the high capacity component is greatly increased, due to a higher K. The increase in influx predicted was not as large as the increase observed in brain tryptophan content. This may be due to the effect of changes in plasma unbound tryptophan which were not taken into consideration in the calculations. The two-fold increase seen during acute hepatic failure (Section II) would undoubtedly contribute to increased tryptophan transport.

The transport of amino acids and their incorporation into protein appear to be intimately related (Lajtha, 1974; Pardridge, 1977).

Serotonin synthesis seems to be even more sensitive to precursor availability possibly because this process is highly localized and does not reutilize tryptophan as occurs in protein metabolism. Therefore alterations in transport of tryptophan and other amino acids, especially those also involved in neurotransmitter synthesis, may have serious consequences on cerebral metabolism and function.

The finding of selective alterations in the blood-brain barrier implies that transport of other substrates may be differently affected. Since several pathways in the brain appear to be substrate-limited, e.g. acetylcholine synthesis (Cohen & Wurtman, 1976), glucose metabolism in some conditions (Betz, Gilboe & Drews, 1976) and ketone-body metabolism (Ruderman, Ross, Berger & Goodman, 1974), an investigation of possible changes in transport mechanisms seems justified in many conditions where brain metabolism is altered.

GENERAL DISCUSSION

A. METABOLIC INTERRELATIONSHIPS

This thesis is concerned with changes in brain metabolism in a condition of altered consciousness. Consciousness is believed to depend on the proper functioning of the reticular activating system of the brainstem and the modifying effect of the cerebral cortex (Breen & Schenker, 1972). A disturbance of consciousness may result from effects on specific areas involved or general effects on brain metabolism as a whole (Plum & Posner, 1972). Since no metabolic pathway has been specifically related to the maintenance of consciousness, all metabolic aspects must be considered. In addition, there are many interrelationships in brain metabolism (Figure 25) and the various systems do not operate independently of one another. Glucose is the source of carbon for most substrates except the essential fatty acids and essential amino acids. Therefore energy metabolism is closely linked to metabolism of the neuroregulator amino acids aspartate, glutamate, glutamine and GABA. These are in equilibrium with intermediates of the Krebs cycle. Indeed it has been suggested that the large glutamate pool functions as a stabilizer for the energy requirements of the brain (Nicklas & Berl, 1973).

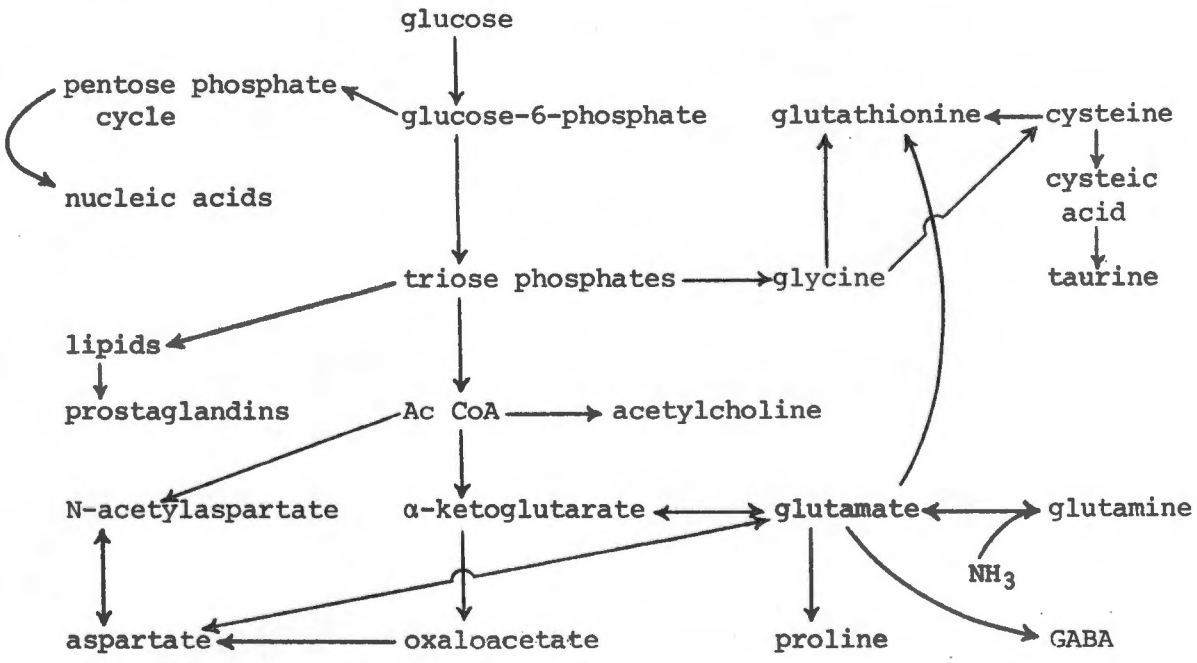
Relationships have also been postulated to exist between energy metabolism, the amino acid neuroregulators and the neurotransmitters derived from the aromatic amino acids, namely the catecholamines and serotonin. For example:

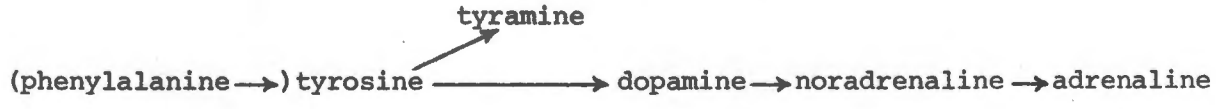
FIGURE 25

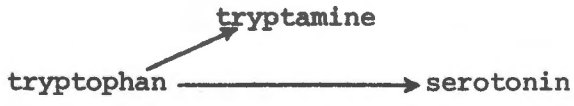
VARIOUS INTERRELATIONSHIPS AMONG COMPOUNDS OF NEUROCHEMICAL INTEREST

(adapted from Cooper et al., 1978)

No direct conversion is necessarily implied by the arrows.







(a) Acids of the Krebs cycle, such as fumarate, stimulate the conversion of dopamine to noradrenaline in vitro (Goldstein, Joh & Garvey, 1968; Levin, Levenberg & Kaufman, 1960).

(b) Ca^{2+} is necessary for both release of neurotransmitters as well as normal metabolism of the glucose-derived amino acids (Berl, Clarke & Nicklas, 1970).

(c) Reserpine stimulates turnover of these amino acids as well as releasing catecholamines by affecting vesicle storage. (These findings led to the suggestion that catecholamines have a role as metabolic regulators in addition to functioning as neurotransmitters [Nicklas, Berl & Clarke, 1974; Nicklas & Berl, 1973]).

(d) Flux of carbon from glucose to amino acids is decreased by hepatectomy, administration of phenylalanine, tryptophan or L-dopa, and when brain concentrations of serotonin and 5-HIAA are increased. It was suggested that aromatic amino acids could affect brain glucose metabolism by competition for co-factors, especially pyridoxal-5-phosphate which is needed for the formation of glutamine, glutamate, aspartate and GABA from Krebs cycle intermediates and also for L-amino acid decarboxylase and tyrosine amino-transferase (Wong & Tyce, 1978).

These interrelationships are not surprising because of the dependence of neuronal function and transmitter release on energy metabolism. Metabolic pathways concerned with specialized brain functions are separated to a certain extent from general metabolism by compartmentation and localization. This enables substances such as aspartate and glutamate, which are involved in intermediary metabolism, to function

as highly active neuroregulators. Similarly, a precursor used for several processes, e.g. tryptophan, (used for both protein and serotonin synthesis) is separated into different pools.

B. NEUROCHEMICAL CHANGES IN HEPATIC ENCEPHALOPATHY

During hepatic encephalopathy, alterations were found in many diverse brain metabolites, as well as in the transport mechanism for tryptophan across the blood-brain barrier. These were qualitatively similar in encephalopathy due to acute or chronic liver failure, and therefore, although the pathogenesis of the two conditions may be very different, the neurochemical changes are discussed together.

In general, the changes seen involved increases in inhibitory substances (GABA, glutamine, tryptophan and therefore probably serotonin), and decreases in excitatory substances (glutamate and aspartate). Since the general state of activity of the brain may be related to the relative levels of excitatory and inhibitory material (Hebb, 1970), these alterations may reflect a disturbance of this balance and thus lead to a disturbance of brain activity.

1. AMINO ACID NEUROREGULATORS

Decreases in brain aspartate or glutamate were found in all conditions examined. There was also an increase in GABA during acute hepatic failure. Since ammonia is associated with the metabolism of these amino acids, the large increase in its brain concentration may influence the pools of these neuroregulator substances and thus contribute to the development of their imbalance. High brain content of ammonia also promotes increased synthesis of glutamine which may have an inhibitory effect by acting as a false neurotransmitter. Increases in glutamine concentrations are of special interest for several reasons:

a. Glutamine synthesis occurs exclusively in glial cells or astrocytes (Benjamin & Quastel, 1972; Martinez-Hernandez et al., 1977) which are the only brain structures to show morphological alterations during hepatic failure (Cavanagh, 1974; Schenker et al., 1974).

b. In addition to possibly having false neurotransmitter activity (Baldessarini & York, 1974), glutamine may influence brain glutamate levels (Bradford, Ward & Thomas, 1978).

c. An important role for glutamine has been suggested in the postulated enzymatic basis of neutral amino acid transport across the blood-brain barrier (Samuels, 1977) which involves the γ-glutamyl cycle and the enzyme γ-glutamyl transpeptidase (Meister, 1974; Orlowski, Sessa & Green, 1974). In the original hypothesis, γ-glutamyl transpeptidase was believed to catalyse the transfer of the γ-glutamyl residue of glutathione to an amino acid at the blood-brain barrier. The γ-glutamyl-amino acid is then translocated into the cell and the amino acid released and eventually glutathione re-formed. γ-Glutamyl transpeptidase is present at high concentrations in the choroid plexus and in brain capillaries (Tate, Ross & Meister, 1973; Shine, Hertz, Suddith & Haber, 1979; Orlowski et al., 1974). Its activity increases after birth, concomittantly with the development of the blood-brain barrier (Sessa & Perez, 1975). There were several objections to this proposal; primarily the high energy requirements of the pathway and the low specificity of γ-glutamyl transpeptidase. In addition, the phenomenon of exchange diffusion

could not be accounted for by the pathway. To overcome these problems a second step in the operation of the cycle was postulated (Samuels, 1977). It was suggested that the donor compound is γ-glutamyl-glutamine and that glutamine is exchanged for the entering amino acid. If the supply of intracellular glutamine is adequate, regeneration of γ-glutamyl-glutamine means that only a low expenditure of energy is required. Since the lipoprotein form of γ-glutamyl-transpeptidase is so firmly bound to the membrane, it was further suggested that access to this enzyme is dependent on the relative lipophilia of the entering large neutral amino acids. In this way a membrane sequestered enzyme of low specificity could mediate transport of neutral amino acids. Other free amino acids may be used in place of glutamine, thus accounting for the exchange phenomenon. Recently it was found that administration of an inhibitor of the γ-glutamyl cycle, methionine sulphoximine, resulted in a reduced rate of transport of tyrosine and valine from blood to brain (Samuels, Fish & Freedman, 1978). (Methionine sulphoximine, which inhibits glutamine synthesis in addition to one of the enzymes in the γ-glutamyl pathway, also reduces toxicity from ammonia [Warren & Schenker, 1964].) These results provided further support for the hypothesis.

This postulated role for glutamine in blood-brain barrier transport of the neutral amino acids may suggest a link between two findings in hepatic encephalopathy, namely increased brain glutamine content and increased tryptophan transport, as discussed further below.

2. AMINE NEUROREGULATORS AND PLASMA AMINO ACIDS

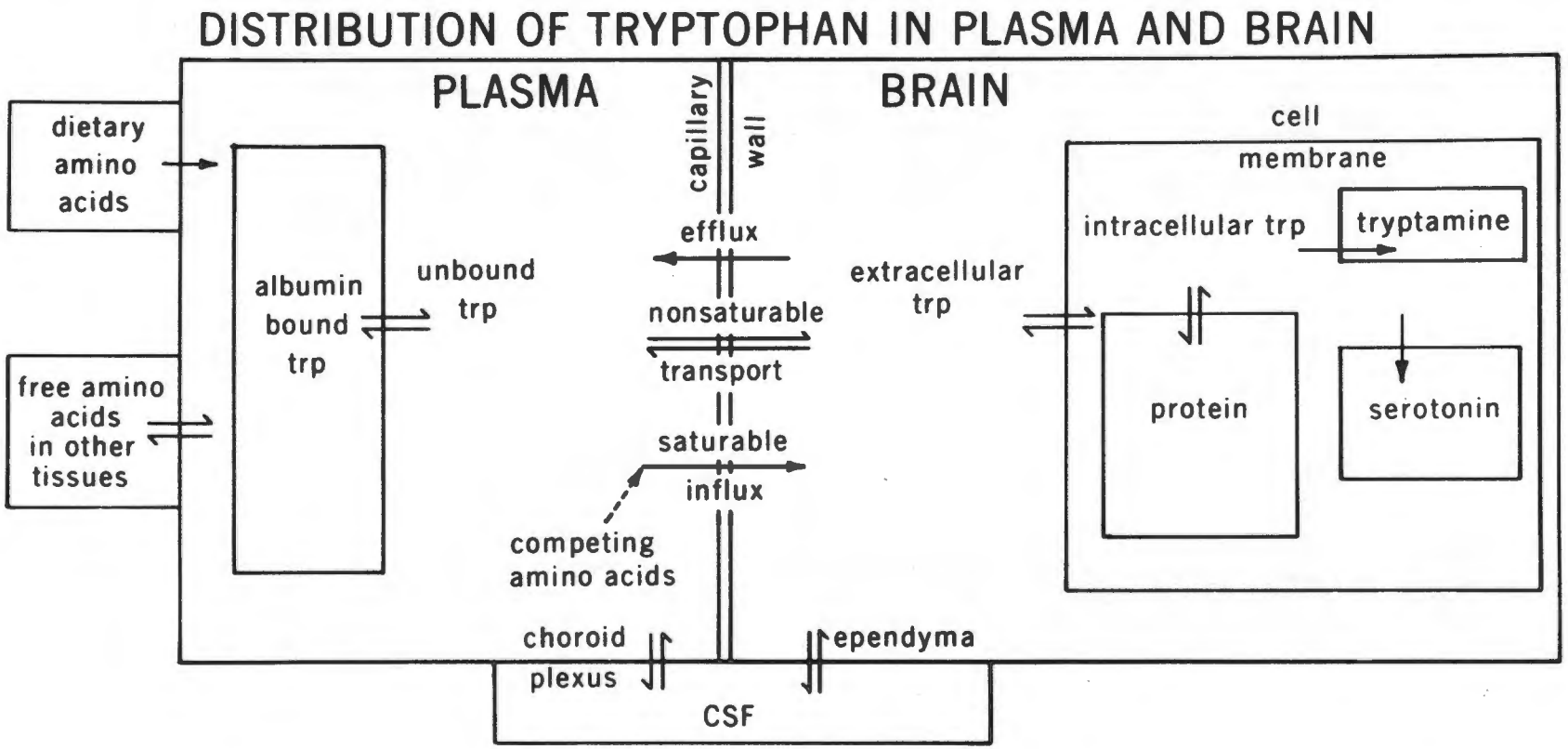
The aromatic amino acid tryptophan appears to have special importance in brain function. The synthesis of the neurotransmitter serotonin, and possibly protein synthesis as well, is limited by tryptophan concentrations (Friedman et al., 1972; Roberts, 1974). High levels of tryptophan have also been shown to inhibit catecholamine synthesis (Andrews et al., 1978). Together with the other two aromatic amino acids which were consistently increased in brain during hepatic failure, phenylalanine and tyrosine, tryptophan may influence metabolism of glucose to the glutamate-related amino acids as described above. Therefore the significant correlation between changes in brain tryptophan concentration in the various states studied and changes in behaviour and apparent brain dysfunction is very significant. No other metabolite studied shows a similar high correlation. These findings provide further support that tryptophan metabolism may be involved in the development of hepatic encephalopathy.

When whole brain tryptophan is measured, this reflects free tryptophan in cells, interstitial fluid and cerebrospinal fluid, as well as in contaminating blood. Most of the tryptophan in brain tissue (excluding blood) is believed to be intracellular, since extracellular fluid concentrations are very low (Bito et al., 1966). Several factors are involved in determining the concentration and distribution of tryptophan in the brain (Figure 26).

a. Plasma tryptophan binding

Tryptophan is the only amino acid found in plasma both free and bound to albumin (McMenamy et al., 1957). This reversible binding affects 80-90% of circulating tryptophan in the fed state.

FIGURE 26


DISTRIBUTION OF TRYPTOPHAN IN PLASMA AND BRAIN
PLASMA
BRAIN
capillary
wall
dietary amino acids
free amino acids in other tissues
albumin bound trp
unbound trp
efflux
nonsaturable transport
saturable influx
competing amino acids
extracellular trp
cell membrane
intracellular trp
tryptamine
protein
serotonin
choroid plexus
ependyma
CSF

It has been shown that tryptophan is removed from albumin on passage through the brain (Yuwiler, Oldendorf, Geller & Braun, 1977). In this last study the "effective" tryptophan concentration in tryptophan-albumin solutions (i.e., that required to account for the observed influx) was found to greatly exceed the measured free tryptophan and approximate half or more of the total tryptophan concentration. This suggests that rapid re-equilibration between bound and unbound tryptophan occurs in the brain capillaries.

Many conditions in which the dissociation constant of albumin binding is affected by changing plasma free fatty acid concentrations, have been shown to influence whole brain tryptophan content (for examples, see p.117). However, probably because of the dynamic situation described above, direct correlations between unbound and brain tryptophan concentrations have often not been found, e.g. after carbohydrate intake, when brain tryptophan rises even though the resulting insulin secretion causes decreased plasma unbound tryptophan (by decreasing free fatty acids) (Madras et al., 1973, 1974; Fernstrom et al., 1976). In the present studies there were large parallel increases in both unbound plasma tryptophan and brain tryptophan, while total plasma tryptophan decreased, suggesting that the change in unbound tryptophan had some influence on entry into the brain.

b. Blood-brain barrier transport

A consequence of the low K_m of the neutral amino acid transport system is that at normal physiological concentrations, amino acids which share this system competitively inhibit one anothers'

uptake. (Since the K_m values of other tissues are at least 10-fold normal plasma amino acid concentrations, competition affects at these sites are probably not important [Pardridge, 1977].) The effects of competing amino acids on tryptophan transport into the brain have been extensively studied in normal animals (Fernstrom et al., 1976; Gessa et al., 1974; Colmenares et al., 1975; Perez-Cruet et al., 1974; Etienne et al., 1976). Increasing the plasma competitors (within physiological limits) in these experiments, either by dietary means or infusions, resulted in lowered brain tryptophan uptake or content. Fernstrom et al. have repeatedly found positive correlations between brain tryptophan and the ratio of plasma total tryptophan to the sum of the competing amino acids. The hypothesis implicating altered plasma amino acid patterns in the development of hepatic encephalopathy (Munro et al., 1975; Soeters & Fischer, 1976), is based on these studies performed in normal rats. From the results described in this thesis it is clear that such extrapolations from the normal condition to that of hepatic encephalopathy cannot be made. As is most clearly evident in the case of acute hepatic failure, the plasma amino acid pattern, if it were the only factor involved, would have resulted in decreased brain tryptophan content rather than the increase observed. In chronic hepatic failure the plasma amino acid pattern obtained in the present study was similar to that described by others and used as a basis for the hypothesis. However, as discussed in Results Section III, this pattern would not result in a change in the apparent K_m of transport sufficient to result in increased tryptophan influx, and again an alteration in the

transport mechanism itself was suggested to account for the high brain tryptophan concentrations.

Studies of tryptophan transport itself have been restricted to measurements of the rates of the unilateral influx of tracer quantities of tryptophan in single-injection (Pardridge & Oldendorf, 1975a) or steady-state methods (Pratt, 1976). With the exception of the branched chain amino acids, the net uptake of amino acids by brain (determined from arteriovenous differences) is not significantly different from zero (Betz & Gilboe, 1973). Net uptakes are lower than influx values because in the steady state, influx of amino acids into brain and subsequent metabolism is almost balanced by exit of amino acids from brain to blood. When competitors normally present in the circulation are taken into account, values for influx obtained by single injection techniques are considerably lower (Table 26, 27) and there is less difference between these influx values and net uptake. It is likely that extensive reutilization of tryptophan within the brain obviates the need for entry of large quantities of plasma tryptophan. Efflux systems are difficult to demonstrate experimentally. Since the ECF concentrations of amino acids are much lower than plasma or intracellular concentrations (Bito et al., 1966), a facilitated diffusion mechanism such as the one which mediates influx and which is equilibrative, could not mediate the exit of the amino acids against a concentration gradient. Active efflux systems, requiring energy and able to work against concentration gradients, have been described for organic anions (Pardridge & Oldendorf, 1977) and more recently for the

nonessential amino acids glycine, alanine and serine (Betz & Goldstein, 1978), which are transported only to a very limited extent from blood to brain (Oldendorf, 1971b). Efflux may also be mediated by the process of counterdiffusion or hetero-exchange by which the entry of one substrate drives the exit of the same or related substrate. This process is characteristic of facilitated diffusion mechanisms in isolated systems from the brain such as slices or cells (Battistin, Piccoli & Lajtha, 1972; Christensen, 1969) and could take place as part of the γ-glutamyl-glutamine pathway as described above. Consideration of this phenomenon raises the possibility that increased brain concentrations of substrates could actually cause increased transport into the brain instead of the opposite as usually assumed. This has been examined in the situation of decreased glucose transport during hypoxia and found not to be the case (Betz et al., 1976).

Alterations in the blood-brain barrier in pathological conditions have only recently received attention (Livingstone, Potvin, Goresky, Finlayson & Hinchey, 1977; Cremer et al., 1977; James et al., 1978). They appear to be of vital importance during hepatic failure, and may therefore be significant in other conditions. The changes found during hepatic failure are further discussed below.

c. Cell membrane transport

Brain cell membrane transport of tryptophan has been studied in slices, synaptosomes and cultured glial cells (Kiely & Sourkes, 1972; Denizeau & Sourkes, 1977; Parfitt & Graham-Smith, 1974; Bauman et al., 1974). Two saturable transport systems were found,

one low and one high affinity system. Transport was active (concentrative) and dependent on temperature and energy but not Na^+. Stereospecificity and exchange diffusion have been demonstrated in slice and synaptosome preparations (Battistin et al., 1972; Parfitt & Smith, 1974). Active uptake by brain slices of the aromatic amino acids appeared to be unsaturated by extracellular concentrations (Oja, Lähdesmäki & Vahvelainen, 1974). In these experiments competitive inhibition among the aromatic amino acids was also demonstrated. Since the reported rate of cell uptake is several-fold higher than the estimated rate of influx of tryptophan across the blood-brain barrier, it is thought that cell transport is probably not rate-limiting in the overall transport of tryptophan from the plasma to brain cells (Pardridge & Oldendorf, 1977).

Glial cell transport of tryptophan may be very important because brain capillaries are surrounded by glial endfeet and it is likely that substrates must pass through these cells before reaching neurons.

d. Intracellular metabolism

Intracellular tryptophan is used mostly for protein synthesis. It has been estimated that up to 400 times as much aromatic amino acid is used for protein as for neurotransmitter synthesis (Barondes, 1974) even when the very short turnover time of the monoamines compared to that of the proteins is taken into acount. (The concentrations of the minor amines such as tyramine, tryptamine and phenylethylamine constitute only 2-5% of the monoamines [Pardridge, 1977]).

Synthesis of serotonin is anatomically separated from most of the protein synthesis in the brain in two ways:

(1) serotonin synthesis is confined to a small proportion of neurons that contain tryptophan hydroxylase.

(2) most of the synthesis is believed to occur in the nerve terminals whereas protein is synthesized almost exclusively in cell bodies (only 1% of the synthetic enzymes are present in the cell bodies where they themselves are made).

This segregation of serotonin and protein synthesis prevents competition for tryptophan between the two processes in whole brain. In serotoninergic neurons such competition has not been excluded. Amino acids for protein synthesis occur in several pools (Roberts, 1974). Since tryptophan appears to be the limiting amino acid for protein synthesis in other tissues such as the liver (Munro, 1970) the same may be true of brain protein synthesis. In general, the supply of essential amino acids does influence the rate of cerebral protein synthesis (Roberts, 1968). Thus, the abnormal quantities and proportions of the brain amino acids found during hepatic encephalopathy may conceivably affect protein synthesis.

The cell bodies of the neurons containing serotonin are restricted to nine clusters in the raphe regions of the pons and upper brain stem (Cooper et al., 1978). Areas innervated by these neurons include the cortex, striatum, cerebellum and hypothalamus. Serotonin synthesis is believed to take place in the nerve terminals to which tryptophan could be supplied by membrane transport into the terminals or by axoplasmic flow from the cell body. Axoplasmic

flow has been shown for some amino acids and proteins (Pardridge, 1977). As described in detail in Results Section II, there is considerable direct and indirect evidence for the dependence of serotonin synthesis on brain tryptophan content. In many conditions where the concentration of tryptophan is increased, the cerebral turnover of serotonin is also increased (Gessa & Tagliamonte, 1974; Tagliamonte et al., 1971, 1973a; Guierinot et al., 1974; Pérez-Cruet et al., 1971; Messing et al., 1976; Colmenares et al., 1975; Curzon et al., 1972, 1973b). Evidence that an increase in tryptophan in whole brain is also reflected in serotoninergic neurons was provided by work showing that single raphe neurons in the brain stem fluoresced more brightly (indicating increased serotonin content) when animals were injected with large doses of tryptophan (Aghajanian & Asher, 1971). These data emphasize the significance of altered tryptophan transport and brain metabolism in the development of hepatic encephalopathy.

It is clear from the above that many transport and metabolic steps could be involved in regulating the utilization of tryptophan. However, there is much evidence that transport across the blood-brain barrier is of primary importance in determining the ultimate fate of tryptophan in the brain as well as whole brain content as it is usually measured. The significant correlation obtained between tryptophan transport rates and brain tryptophan content described in Results Section III supports this statement.

3. BLOOD-BRAIN BARRIER CHANGES

Until recently it was assumed that no matter what changes are occurring in blood or brain, the transport mechanisms of the blood-brain barrier operate exactly as they do under normal conditions. The present studies showing altered tryptophan transport during hepatic encephalopathy, together with earlier reports (Cremer et al., 1977; Livingstone et al., 1974, 1977) suggest that this assumption may often not be true. Transport across the blood-brain barrier appears to be limiting in the utilization of metabolites by the brain in several pathways as discussed above, therefore any alterations in the mechanisms controlling transport could have significant effects on brain metabolism and therefore function.

The alterations in tryptophan transport found during hepatic encephalopathy cause an increased rate of transport which clearly contributes to the increased content of brain tryptophan found during hepatic failure. The demonstration of a nonsaturable component of transport, which contributed considerably to the total influx, may be of special significance, since this component is unaffected by competing amino acids. The findings could thus explain the apparent paradox of increased brain tryptophan in the presence of very high plasma amino acids in acute hepatic failure.

The changes found in the blood-barrier appeared to be selective and thus the effect on transport of other substrates cannot be predicted. The large increase in brain content of tyrosine and phenylalanine may suggest that entry of these amino acids is increased as well as that of tryptophan, since these all share the same transport mechanism. At this stage it is unknown whether the alterations in the blood-brain

barrier are entirely the result of some pathological change, or evidence of a regulatory process responding to abnormal substrate concentrations either within the brain or in the plasma. (Increased influx of ketone bodies into the brain has been found after prolonged ketonemia [Gjedde & Crone, 1975].) In both cases the changes could further contribute to the development of the condition.

These studies emphasize the sensitivity of brain metabolism to blood metabolite levels, through its dependence on substrate transport. It is becoming clear that the brain is more responsive to changes in blood than was thought when the term "blood-brain barrier" was first used. Furthermore, these studies demonstrate the importance of investigating transport of precursors into brain in addition to metabolite changes within the brain for a fuller understanding of the neurochemical changes occurring during hepatic encephalopathy and very possibly other conditions where brain function is disturbed.

C. COMPARISON WITH OTHER METABOLIC COMAS

The neurochemistry of other metabolic comas, with the exception of hypoglycaemic coma, has not been as extensively studied as that of liver failure. During hypoglycaemic coma there is a general decrease in brain glycolysis with depletion of glutamine, glutamate and alanine and an increase in aspartate and later ammonia (Goldberg, Passoneau & Lowry, 1966; Lewis, Ljunggren, Norberg & Siesjö, 1974; Ferrendelli, 1974, 1975; Tews, Carter & Stone, 1965; Norberg et al., 1975; Norberg & Siesjö, 1976; Gorell, Dolkart & Ferrendelli, 1976). However the cause of disturbed consciousness remains unknown, since, as with hepatic coma, brain function appears to decrease before there is a shortage of energy intermediates (Ferrendelli & Chang, 1973; Lewis et al., 1974; Gorell, Law, Lowry & Ferrendelli, 1977).

Changes in tryptophan metabolism similar to those in hepatic encephalopathy have been reported in uraemic coma. Reduced total plasma tryptophan and increased unbound tryptophan have been found both in patients with chronic renal failure (De Torrente, Glazer & Gulyassy, 1974; Sullivan, Murnoghan, Callaghan, Kantamaneni & Curzon, 1978) and in rats with experimental chronic renal failure (Siassi, Wang, Kopple & Swendseid, 1977). Raised serotonin and reduced dopamine levels were found in postmortem brains of uraemic coma patients (Jellinger, Irsigler, Kothbauer & Riederer, 1977), while increased 5-HIAA was found in the cerebrospinal fluid of patients and in the brains of rats with chronic renal encephalopathy (Sullivan et al., 1978; Siassi et al., 1977). These findings suggest that disturbed neurotransmitter - and especially serotonin metabolism, may play an

important role in the development of uraemic as well as hepatic encephalopathy. Altered neurotransmitter metabolism is also thought to be involved in hypoglycaemic coma (Duffy & Plum, 1976).

In most studies of brain metabolism during encephalopathic conditions, including hepatic encephalopathy, whole brain metabolite content was studied. This has the disadvantage that changes in specific regions or small but important pools may be masked and therefore not detected. However the large changes seen in glutamine and tryptophan during hepatic encephalopathy, for example, although probably not reflecting the small glial and serotonin precursor pools respectively, very possibly affect these pools. Ideally, turnover of the various substrates, which may be more closely associated with brain function, should be determined in addition to their concentrations. However such studies are much more difficult to carry out experimentally. It is possible that investigations of specific brain regions may uncover more changes that are shared by the various types of metabolic coma.

One feature common to several types of coma including those due to hepatic failure, kidney failure, hypoglycaemia, hypoxia, as well as posttraumatic coma and coma induced by various chemical agents (barbiturates, fatty acids, dimethylsulphide), is that transmission failure precedes energy failure (Brodersen, 1974; Siesjö, Jóhannsson, Norberg & Salford, 1975; Berghof et al., 1970; Derr & Zieve, 1973; Ferrendelli, 1975). This suggests that in the cases where there is decreased availability of essential substrates, there is a protective mechanism which initially reduces brain activity to preserve energy, possibly for more essential life-sustaining processes, or that transmission is directly affected by the condition.

Blood-brain barrier transport does not appear to have been investigated in conditions of disturbed brain function other than hepatic encephalopathy or hypoxia (Betz et al., 1976).

D. HYPOTHESIS

The pathogenesis of hepatic encephalopathy appears to depend on many factors. Instead of searching for a single causative agent it may therefore be necessary to consider multiple events which may be linked at the cellular and subcellular levels. Taking into consideration the neurochemical alterations described in this thesis a sequence of events leading to brain dysfunction in hepatic failure may be put forward as follows. Shunting of portal blood past the failing liver leads to high blood concentrations of amino acids, especially the aromatic amino acids tyrosine, phenylalanine and unbound tryptophan, as well as ammonia from other nitrogenous substances. When liver necrosis is present even more aromatic amino acids are released into the circulation. There is a change in the blood-brain barrier transport mechanism for the neutral amino acids which leads to increased transport of tryptophan and possibly phenylalanine and tyrosine, thus causing higher brain content of these amino acids. The change in the transport mechanism may be caused by the abnormal plasma amino acid pattern, i.e. an induction or regulatory process. Alternatively, increased entry of ammonia from the blood into the glial cells surrounding the brain capillaries may promote increased synthesis of glutamine which in turn may stimulate activity of the γ-glutamyl-glutamine pathway for transport of the neutral amino acids.

Increased brain tryptophan leads to increased serotonin synthesis, and both tryptophan and phenylalanine may inhibit catecholamine synthesis at the aromatic-L-amino acid decarboxylase step. The aromatic

amino acids can also interfere with the metabolism of the amino acid neuroregulators from glucose. The overall result of these changes is a disturbance of the balance between excitatory and inhibitory neuroregulators in the brain, i.e. decreased catecholamines, asparate and/or glutamate, and increased serotonin and GABA. High glutamine concentrations may also have an inhibitory effect. Such an imbalance in the neuroregulators then produces alterations in neuronal activity necessary for the normal maintenance of consciousness by acting on neuronal membranes. For example, serotonin is thought to cause increased Ca^{2+} inside neurons by increasing membrane permeability to calcium, as well as stimulating adenyl cyclase resulting in increased intracellular cAMP, which promotes Ca^{2+} release from mitochondria. A rise in Ca^{2+} lowers excitability by raising the membrane permeability to K^{+} (Krnjević, 1975; Rasmussen, 1975).

In this way the various neurochemical changes which appear to be closely associated with the development of hepatic encephalopathy, may be part of a cascade of events ultimately resulting in decreased neuronal activity and brain function.

E. CONCLUSIONS

Several main conclusions can be drawn from the preceding discussion and earlier sections, and these are summarized below.

ANIMAL MODELS

1. The three different methods used to produce chronic and acute hepatic failure satisfy the biochemical requirements for these conditions. However more sophisticated behavioural monitoring is needed to provide clearer evidence of encephalopathy and coma.

2. Comparison of the results from the surgically and galactosamine-induced acute hepatic encephalopathy confirmed that the plasma and brain changes observed were the result of acute liver failure. The use of galactosamine has some advantages over the surgical model.

CHANGES IN NEUROREGULATORS AND ASSOCIATED METABOLITES

1. Hepatic encephalopathy is associated with an imbalance in the brain content of neuroregulators, in the direction of an increase in those with inhibitory actions and a decrease in those with excitatory actions. The changes observed were more pronounced in acute hepatic failure, especially those involving tryptophan and ammonia. The most striking difference between the chronic and acute conditions was in the plasma amino acid concentrations, with very large increases in acute hepatic failure and only slight changes in chronic hepatic failure.

2. Glutamine changes may be of special importance due to the alterations observed in glial cells (where glutamine is exclusively

synthesized), their proximity to the brain capillaries and the changes seen in the blood-brain barrier, and the possible involvement of glutamine in barrier transport.

3. The importance of hydroxy-indole involvement in encephalopathy is confirmed by the correlation of changes in brain tryptophan content with the various stages of hepatic failure and encephalopathy.

4. With respect to the factors contributing to the increased brain tryptophan content found during hepatic failure, it is clear that at least three must be considered: plasma competing amino acids, plasma unbound tryptophan, and the kinetics of the transport system.

BLOOD-BRAIN BARRIER CHANGES

1. The alterations found in the kinetics of the tryptophan transport mechanism result in increased tryptophan influx across the blood-brain barrier and contribute to the increased brain tryptophan content.

2. The changes appear to be selective, therefore there may be different changes in other barrier transport mechanisms transporting other essential substrates into the brain.

3. It is suggested that transport mechanisms may also be affected in other pathological conditions, including those involving brain dysfunction.

4. These possibilities suggest that caution should be used in extrapolating from the normal to pathological conditions and therefore in the suggested use of intravenous amino acids, other dietary precursors and drugs in the treatment of brain disease.

INTERRELATIONSHIPS

The work in this thesis emphasizes several facets of the neurochemistry of a pathological condition.

1. Brain metabolism of the amino acid and aromatic neuroregulators appears to be interrelated at many levels, which must be taken into account when searching for precipitating events in the development of the condition.

2. The dependence of neuroregulator synthesis on precursor availability stresses the importance of plasma substrate distributions and especially blood-brain barrier transport in influencing brain function.

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